CHARACTERIZATION AND CONTROL OF PSYCHROTOLERANT BACTERIAL SPOILAGE ORGANISMS ASSOCIATED WITH PASTEURIZED FLUID MILK

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Stephanie Noelle Masiello

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Stephanie Noelle Masiello, Ph.D.

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Millions of gallons of pasteurized fluid milk meant for consumption in the U.S. are discarded. Bacterial spoilage is the largest contributing factor for product loss of pasteurized fluid milk. Microbes can be present in pasteurized milk via two main routes: (i) survival of pasteurization by bacteria present in raw milk (generally Gram-positive sporeformers), and (ii) post-pasteurization contamination (PPC) of the product. This product spoilage is further complicated when spoilage microbes can survive refrigeration temperatures and even grow in the cold storage climate. The studies presented here focused on ways to achieve higher quality, longer lasting pasteurized fluid milk by exploring (i) the application of molecular technologies to better understand and potentially track psychrotolerant coliforms responsible for post-pasteurization contamination of fluid milk and (ii) the associations between psychrotolerant sporeforming spoilage organism presence in fluid milk and dairy farm management practices. Our data revealed that psychrotolerant coliforms introduced as PPC in fluid milk have considerable taxonomic and phenotypic diversity, indicating that hygienic issues within a fluid milk processing plant may lead to introduction of a diverse group of coliform contaminants capable of having a direct impact on pasteurized milk quality and the consumer's sensory experience. Our cross-sectional study identified dairy farm management practices related to milking time hygiene may simultaneously lower bulk tank somatic cell count on dairy farms as well as psychrotolerant sporeformer levels in bulk tank milk, suggesting that on-farm adjustments in management specifically focused on udder cleanliness may directly impact the shelf-life of pasteurized fluid milk. Finally, we showed through our longitudinal study that dairy

farm management practices related to overall farm cleanliness were associated with a decrease in the presence of psychrotolerant Bacillales spores in bulk tank milk after 21 days at 6°C post-heat treatment. The combined results from the cross-sectional and longitudinal studies indicate that on-farm adjustments in management focused on both general farm and cow cleanliness may have a direct impact on psychrotolerant Bacillales spore presence and therefore impact the shelf-life of pasteurized fluid milk. The studies presented here contribute to our understanding of psychrotolerant spoilage organisms isolated from fluid milk and provide insights into potential control strategies aimed at achieving high quality, longer lasting pasteurized fluid milk.

BIOGRAPHICAL SKETCH

Stephanie Noelle Masiello was born on July 25, 1985 in Norwalk, CT as an only child to Anthony Masiello and Joanne Mallie Masiello. Stephanie graduated from Bryn Mawr College with a Bachelors of Art in Classical Cultures and Societies in 2007. During her undergraduate career, Stephanie also pursued science classes to fill an interest in large animal veterinary medicine. This led to her completing a Masters of Science degree in Dairy Science from Virginia Polytechnic University in 2010. During her time at Virginia Tech, Stephanie conducted her Masters research studying dairy cow mastitis and *Enterococcus* spp. Stephanie began as a doctoral student at Cornell University in Food Science & Technology in August 2010. She is currently an epidemiologist at the Chicago Department of Public Health and resides in northern Chicago with her husband Dave, son Connor, and dog Orzo.

Dedicated To My Family

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CHAPTER 1

INTRODUCTION

As described in the 2017 United Nations World Prospect Report, the world population will total an estimated 9.8 billion people by 2050 and the United States of America will be part of a nine-country cohort accounting for half of the population growth observed between 2017 and 2050 (United Nations, 2017). The future pressure to produce long-lasting, nutrient-rich food may prove challenging for US agriculture industry and is intensified by the current challenge of combating food loss, especially in products with short shelf-lives. Of the > 5 billion gallons of pasteurized fluid milk meant for consumption in the U.S. every year, one-fifth is discarded by consumers and foodservice businesses (IDFA, 2010; Gunders, 2012). Bacterial spoilage is the largest contributing factor for product loss of pasteurized fluid milk (Boor, 2001; Durak et al., 2006). Microbes can be present in pasteurized milk by two primary routes: (i) survival of pasteurization by bacteria present in raw milk (generally Gram-positive sporeformers), and (ii) post-pasteurization contamination (PPC) of the product (Boor and Murphy, 2002; Huck et al., 2007a). This product spoilage can be further complicated when spoilage microbes can not only survive at temperatures used for refrigeration (often used to increase shelf-life), but actually thrive.

Gram-positive *Bacillus* spp. and *Paenibacillus* spp. form heat-resistant spores that can withstand high temperature short time (HTST) pasteurization commonly used for fluid milk processing (Collins, 1981; Fromm and Boor, 2004; Ranieri et al., 2009). The ability of these organisms to survive heat treatment and of certain strains to grow at refrigerated storage temperatures results in milk spoilage (Washam et al., 1977; Huck et al., 2008). Reduction or elimination of these bacterial contaminants can result in extension of fluid milk shelf-life, which

would continue to enhance the dairy industry by providing overall higher product quality. However, eliminating sporeforming bacteria is challenging, as these organisms have been isolated from the dairy farm environment (Vaerewijick et al., 2001; te Giffel et al., 2002; Scheldeman et al., 2002; Scheldeman et al., 2004). Sporeformers have also been isolated along the dairy product processing continuum, from milk trucks to packaged final products (Ranieri et al., 2009; Martin et al., 2011). Identification of the same bacterial subtypes in both raw and pasteurized milk samples suggests that pasteurized fluid milk spoilage can result from bacteria that enter raw milk on the farm (Huck et al., 2007b). Thus, there is a need to (i) explore associations between on-farm practices and resulting levels of sporeformers, (ii) identify potential mitigation touch-points, and (iii) assess the feasibility of these farm-based mitigation strategies.

To combat spoilage organisms and achieve high fluid milk quality, attention must be focused not only on the farm production environment, but also within the milk processing environment. Preventing PPC with spoilage microorganisms remains a major challenge for dairy processors (Ralyea et al., 1998; Ranieri and Boor, 2009; Martin et al., 2011). Along with *Pseudomonas* spp., coliform bacteria are frequently isolated PPC contaminants in pasteurized fluid milk (Martin et al., 2011). Detection of coliforms is important in the dairy industry as they are frequently used as hygiene indicators. Furthermore, there are clear regulatory limits for the presence of coliforms in finished dairy products (FDA, 2011). Coliform bacteria that are psychrotolerant and capable of growing at refrigerated storage temperatures are of particular concern for the dairy industry, as psychrotolerant growth can result in physical degradation and unacceptable sensory characteristics of the product due to the production of lipolytic and proteolytic enzymes (Nörnberg et al., 2010). While coliform testing continues as common

practice in the dairy industry, the available data on the phenotypic and genotypic coliform diversity found in dairy products are surprisingly limited (Eneroth et al., 1998; Juven et al., 1981; Wessels et al., 1989). An improved understanding of the diversity and spoilage potentials of fluid milk associated coliforms is vital to improve fluid milk quality. It will also provide opportunities for better baseline data on coliform diversity associated with fluid milk, validate more robust and faster detection methods, and may also facilitate source tracking of coliform contamination.

The following chapters will expand on the current knowledge of psychrotolerant bacterial spoilage organisms associated with pasteurized fluid milk and provide feasible interventions that could improve pasteurized fluid milk quality. Specifically, utilizing current molecular technologies to better understand psychrotolerant coliform diversity in pasteurized fluid milk may lead to simple, efficient, and targeted surveillance for these spoilage organisms within fluid milk processing facilities. Additionally, understanding how specific management decisions made on dairy farms may impact the level of psychrotolerant sporeformers in bulk tank milk may allow farmers to achieve a higher quality product by simply reviewing current protocols instead of implementing new or expensive technologies.

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CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF PSYCHROTOLERANT COLIFORM BACTERIA ISOLATED FROM PASTUERIZED FLUID MILK

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ABSTRACT

The presence of coliform bacteria in pasteurized fluid milk typically indicates that product contamination occurred downstream of the pasteurizer, but may also indicate pasteurization failure. While coliform detection is frequently used as a hygiene indicator for dairy products, our understanding of the taxonomic and phenotypic coliform diversity associated with dairy products is surprisingly limited. Therefore, using Petrifilm Coliform Count plates, we isolated coliforms from HTST-pasteurized fluid milk samples from 21 Northeast US fluid milk processing plants. Based on source information and initial characterization using partial 16S rDNA sequencing, a total of 240 non-redundant isolates were obtained. The majority of these isolates were identified as the genera *Enterobacter* (42% of isolates), *Hafnia* (13%), *Citrobacter* (12%), *Serratia* (10%), and *Raoultella* spp. (9%); additional isolates were classified into the genera *Buttiauxella*, *Cedecea*, *Kluyvera*, *Leclercia*, *Pantoea*, and *Rahnella*. A subset of 104 representative isolates was subsequently characterized phenotypically. Cold growth analysis in Skim Milk broth showed that all isolates displayed at least a 2 log increase over 10 days at 6°C; the majority of isolates (n = 74) displayed more than a 5 log increase. A total of 43% of the representative isolates displayed lipolysis when incubated on Spirit Blue agar at 6°C for 14 days, while 71% of isolates displayed proteolysis when incubated on Skim Milk agar at 6°C for 14 days. Our data indicate that a considerable diversity of coliforms is found in HTST-pasteurized

fluid milk and that a considerable proportion of these coliforms have phenotypic characteristics that will allow them to cause fluid milk spoilage.

Key words: coliform, HTST pasteurized milk, milk spoilage, cold growth

INTRODUCTION

Preventing post-pasteurization contamination (PPC) with spoilage microorganisms remains a major challenge for dairy processors (Ralyea, R. D., M. Wiedmann, and K. J. Boor, 1998; Ranieri and Boor, 2009; Martin et al., 2011). Along with *Pseudomonas* spp., coliform bacteria are frequently isolated PPC contaminants in pasteurized fluid milk (Martin et al., 2011). Coliforms are defined as aerobic/facultatively anaerobic, Gram-negative, non-sporeforming rods capable of fermenting lactose resulting in gas and acid production within 48 h at 35°C (Sperber and Doyle, 2009; Nörnberg et al., 2010). Traditionally, coliforms were considered to be represented by four genera; *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* (Bergey et al., 1939). Today, it has been recognized that over 20 different bacterial genera include strains that have phenotypic characteristics that classify them as coliforms (Imhoff, 2005). Detection of coliforms plays an important role in the dairy industry as coliforms are frequently used as hygiene indicators and as there are clear regulatory limits for the presence of coliforms in finished dairy products. For example, the US Pasteurized Milk Ordinance (PMO) limits the number of coliforms in pasteurized grade "A" milk to ≤ 10 cfu/mL (FDA, 2011).

Coliform bacteria that are psychrotolerant and capable of growing at refrigerated storage temperatures are of particular concern for the dairy industry, as psychrotolerant growth can result in physical degradation and unacceptable sensory characteristics of the product due to the production of lipolytic and proteolytic enzymes (Nörnberg et al., 2010). This is also supported by a recent 10-year study of HTST fluid milk quality in New York State (NYS); milk samples that tested positive for coliforms on the initial day of refrigerated shelf-life had significantly lower sensory scores on day 14 of refrigerated shelf-life compared with samples that were not positive for coliforms on the initial day (Martin et al., 2012). Post-pasteurization contamination

is not isolated to the US; for example, previous research found 40% of fluid milk samples taken from Norway and Sweden had PPC with coliforms (Eneroth et al., 1998).

While coliform testing continues to play an important role in the dairy industry, available data on the phenotypic and genotypic coliform diversity found in dairy products is surprisingly limited. Most reported studies only characterized small sets of coliform isolates and typically these isolates were obtained from only a few processing facilities. For example, researchers in Norway and Sweden assessed coliform levels from 3 different dairy processing plants (Eneroth et al., 1998), while other researchers utilized as little as 6 to as many as 75 coliform isolates to characterize dairy product associated coliform bacteria (Juven et al., 1981; Wessels et al., 1989). An improved understanding of the diversity and spoilage potentials of fluid milk associated coliforms is vital to improve fluid milk quality. For example, better baseline data on coliform diversity associated with fluid milk may facilitate development and validation of more robust and faster detection methods and may also facilitate source tracking of coliform contamination. Therefore, the objectives of this study were thus: (i) to characterize the taxonomic diversity of psychrotolerant coliforms in pasteurized fluid milk obtained from a substantial number of processing plants and (ii) to characterize representative isolates for relevant phenotypic characteristics (i.e., cold growth capability and proteolysis and lipolysis during growth at refrigeration temperatures).

MATERIALS & METHODS

Sample Collection and Handling

Pasteurized milk samples were collected in 2010-2011 from fluid milk processing facilities located in the Northeast US (NYS, Maine, Pennsylvania, Vermont, New Hampshire,

and Massachusetts). All participating processing facilities were enrolled in the Voluntary Shelf-Life (VSL) program, which is administered by the Cornell University Milk Quality Improvement Program (MQIP) (Martin et al., 2012). Processing capacity of facilities ranged from < 1 million to > 600 million lbs of fluid milk annually. Samples collected represented packaged pasteurized products that were processed at each facility via High Temperature Short Time (HTST) pasteurization (at a minimum of 72°C [161°F], 15 s), including whole fat (min. 3.25% milk fat), reduced fat (1.5% or 2% milk fat), lowfat (1% milk fat), and nonfat (< 0.2% milk fat) milk in quart (946 mL), half gallon (1.9 L), or gallon (3.8 L) containers. Containers used for packaging included high-density polyethylene jugs, paperboard cartons, or glass bottles. All milk samples were transported to the MQIP laboratory in coolers packed with ice packs or ice. Samples were held at 4°C until initial testing in the laboratory 24 to 48 h post sample collection. Of the 29 total fluid milk processing plants enrolled in the 2010-2011 VSL program, 21 plants had positive coliform samples and were included in the study reported here.

Coliform Isolation from Milk Samples

Upon arrival at the laboratory, samples were inverted 25 times within 7 s and for each milk sample ~400 mL of milk were aseptically distributed into each of 4 sterile 500mL glass bottles. Bottles were stored at 6°C until the appropriate test day for each bottle. Samples were tested for microbiological and sensory quality on the initial day as well as at 7, 10, and 14 d post-processing (Martin et al., 2012). The initial day was defined as the first day of testing, which varied from 0 to 6 d post-processing due to the plant processing schedule. Samples from a subset of processors with histories of consistently manufacturing high-quality products were also tested at days 17 and 21 post-processing (Martin et al., 2012). Coliform testing was performed on each test day by inoculating milk samples in duplicate on separate Petrifilm Coliform Count plates

(3M, St. Paul, MN); plates were subsequently incubated at 32°C for 24 h according to manufacturer's instructions. For each sample date (e.g., day 7) with a sample positive for coliforms, two coliform isolates were selected from the Petrifilm Coliform Count plates and streaked for purity on Brain Heart Infusion (BHI) agar (Difco, Franklin Lakes, NJ), which was subsequently incubated at 32°C for 24 h. Isolates were confirmed as coliforms by inoculation in Brilliant Green Bile Broth (Difco); only isolates confirmed as coliforms were retained and further characterized. Pure cultures were grown overnight in BHI broth at 32ºC and then frozen in 15% glycerol at -80ºC and stored until further characterization. Prior to further characterization as described below, isolates were streaked for isolation from frozen stock onto BHI agar and grown at 32ºC for 24 h. Detailed information on all isolates is available through Food Microbe Tracker (http://www.foodmicrobetracker.com).

Genus Identification Using Sequencing of Partial 16S rDNA

All isolates were characterized by sequencing a 616 bp fragment of the 16S rRNA gene as described previously (Huck et al., 2008). The resulting 16S rDNA sequence data were used to identify isolates to the genus level. Genus identification was performed using the Ribosomal Database Project (RDP) classifier (Cole et al., 2005); genus identification was assigned based on a seqmatch score (S_ab) of 0.90 or higher (Cole et al., 2005). For five isolates that did not produce 16S rDNA gene products with the protocol reported by Huck et al. (2008), genomic DNA was obtained using the QIAmp DNA Mini Kit (Qiagen, Venlo, the Netherlands) and used for subsequent PCR and sequencing of the 16S rRNA gene as described by Huck et al. (2008).

Partial 16S rDNA data were also used to classify isolates into 16S rDNA sequence types (designated "16SrDNA-STs"). Two isolates were classified into the same 16SrDNA-ST if they

shared an identical partial 16S rDNA sequence. 16SrDNA-ST data were used to select isolates for subsequent phenotypic characterization (i.e., cold growth, proteolytic and lipolytic capabilities, as detailed below). If ≤ 10 isolates were grouped into a given 16SrDNA-ST, a single isolate was selected for phenotypic characterization, while if there were > 10 isolates within a given 16SrDNA-ST, multiple isolates were chosen from that sequence type. With this approach, 104 representative isolates were selected for phenotypic characterization.

Phylogenetic Analysis of Coliform Isolates

To confirm genus identifications for all isolates, a 16S rDNA-based parsimony phylogenetic tree (not shown) was constructed using PAUP (version 4, Sinauer Associates Inc., Sunderland, MA); this tree included the sequences obtained here as well as all type strains of coliforms that were included in the RDP database. Isolates were confirmed as a specific genus if they fell within the same 16SrDNA-ST as the type strain sequence.

Cold Growth Analysis During Refrigerated Storage at 6°C

For cold growth analysis, an isolated colony for a given isolate was inoculated into a tube containing 5 mL of BHI broth (Difco), followed by incubation at 32°C for 24 h. To enumerate the bacteria present after the 24 h incubation, 1 mL of the culture was aliquoted into a 2 mL Eppendorf tube and centrifuged for 10 min at 30,000 rpm. After removal of the supernatant, bacterial pellets were re-suspended with 1 mL of phosphate buffer. A 1 mL aliquot of each suspension was serially diluted with phosphate buffer and a 100 µL aliquot of each sample was plated in duplicate onto Standard Plate Count (SPC) (Difco) agar plates (50 µL per plate) with an AP5000 spiral plater (Advanced Instruments, Inc., Norwood, MA). The remaining volume of the isolate suspension was stored at -20°C for less than 24 h prior to use. Results from

enumeration were used to dilute each remaining suspension with phosphate buffer to a final concentration of 10^2 to 10^3 cfu/mL. A 1 mL aliquot of the final dilution (at 10^2 to 10^3 cfu/mL) was then inoculated into 4 mL of sterile Skim Milk Broth (SMB; Difco); 100 µL of the inoculated SMB were immediately spiral plated onto two SPC agar plates (50 µL per plate) for day 0 bacterial enumeration. Inoculated SMB was subsequently incubated at 6°C for 10 d and bacterial enumeration was performed at days 2, 4, 6, 8, and 10.

Characterization of Proteolytic and Lipolytic Activity in Bacterial Isolates grown at 6°C

The 104 isolates selected for cold growth analyses were further analyzed for ability to produce extracellular proteases and lipases during growth at 6°C. Production of extracellular proteolytic enzymes was determined on Skim Milk Agar (SMA) (Difco) and production of extracellular lipolytic enzymes was determined on Spirit Blue Agar (SB) (Difco) containing an additional pre-prepared lipase reagent representing a mixture of Tributyrin and Polysorbate 80 (Difco). Single bacterial colonies were streaked on the respective plates. After incubation at 6°C for 14 d, isolates were scored for enzymatic activity on a three point scale. For SMA plates, a score of (-) was given to isolates with no visible clearing around the streak area, (+) was given to isolates with noticeable clearing around the streak area, and (++) was given to isolates with an obvious clear zone and opaque zone surrounding the streak area (Frank and Yousef, 2004). For SB plates with the added lipase reagent, a score of (-) was given to isolates with no visible clearing around the streak area, (+) was given to isolates with a small zone of clearing ($\leq$ 2 mm) around the streak area with the remainder of the plate remaining blue, and (++) was given to isolates where the clearing around the streak area was > 2 mm (Immanuel et al., 2008).

Data Analyses

All data were managed in Excel (version 2007, Microsoft, Redmond, WA). Coliform bacteria count data were log transformed prior to analysis and enzymatic activity levels were coded 1 to 3, with 1 = (-), 2 = (+), and 3 = (++). Excel was used to compute the Simpson's Index of Diversity (Simpson, 1949) in order to quantify overall species and 16SrDNA-ST diversity among the 240 non-redundant isolates (isolates were considered redundant if they shared the same plant, date, sample, and genus). SAS statistical software (version 9.3, SAS Institute Inc., Carey, NC) was used to perform Fischer's exact tests to determine whether the proportion of isolates that showed different levels of enzymatic activity differed among genera. JMP Pro statistical software (version 10.0, SAS Institute Inc.) was used to plot bacterial growth over 10 d at 6°C for different isolates representing the most commonly isolated genera.

RESULTS

Coliform Bacteria Isolated from HTST-Pasteurized Fluid Milk Represent Diverse Enterobacteriaceae Genera

A total of 402 coliform isolates obtained from fluid milk samples collected from 21 different fluid milk processing plants in the Northeast US (Table 2.1; Supplemental Table 2.1) were initially characterized by sequencing a 616 bp 16S rDNA fragment. 16S rDNA sequence data were used to (i) classify isolates to the genus level and (ii) assign a 16S rDNA sequence type (16SrDNA-ST) to each isolate; two isolates were classified into the same 16SrDNA-ST if they shared an identical 16S rDNA sequence over the 616 bp sequenced. Genus-level characterization classified the isolates into 11 different Enterobacteriaceae genera, including *Enterobacter*, *Hafnia*, *Citrobacter*, *Serratia*, *Raoultella*, *Buttiauxella*, *Kluyvera*, *Pantoea*, *Cedecea*, *Rahnella*, and *Leclercia* (Table 2.1). The 402 isolates could furthermore be classified into 97 different 16SrDNA-STs.

Table 2.1. Coliform genera isolated from HTST-pasteurized fluid milk samples.

Genus	Number of Isolates	Number of non-redundant isolates[1]	Number of 16SrDNA-STs[2]	Number of plants where a given genus was isolated[3]
Enterobacter	175	100	19	12
Hafnia	52	31	8	6
Citrobacter	45	28	27	12
Serratia	39	24	13	6
Raoultella	38	22	4	5
Buttiauxella	15	11	10	5
Kluyvera	13	9	5	5
Pantoea	11	6	5	3
Cedecea	7	4	2	2
Rahnella	5	4	3	4
Leclercia	2	1	1	1
Total	402	240	97	-

[1]Non-redundant isolates are defined as isolates that did not share the same plant, date, sample, and genus as any other isolate
[2]16SrDNA-ST = 16SrDNA sequence type; two isolates are defined as having the same 16SrDNA-ST if they share an identical partial 16S rDNA sequence
[3]Isolates were obtained from coliform positive samples collected from 21 fluid milk processing plants

The genera that included the largest number of 16SrDNA-STs were *Citrobacter* (27 sequence types), *Enterobacter* (19 sequence types), *Serratia* (13 sequence types), *Buttiauxella* (10 sequence types), and *Hafnia* (8 sequence types) (Table 2.1). The most commonly isolated 16SrDNA-ST within the 402 isolates was *Enterobacter* sp. 16SrDNA-ST 40 (115 isolates) (Supplemental Table 2.1). As multiple isolates from the same fluid milk sample and time in shelf-life were analyzed in a number of cases, classification data were also used to define a set of non-redundant isolates; isolates were considered redundant if they shared the same plant, date, sample, and genus. This approach yielded 240 non-redundant coliform isolates (Table 2.1), which were used to assess the frequency of different coliform genera in HTST-pasteurized fluid milk samples.

To assess the overall genus diversity, Simpson's Index of Diversity (SID) was calculated based on the number of non-redundant isolates classified into each of the 11 genera. Simpson's index at the genus level was 0.78. As the index ranges from 0 to 1 with a larger value indicating greater diversity (Simpson, 1949), our data suggest that the isolates obtained here represent considerable diversity. Hence, phenotypic characterization of these isolates should provide a good representation of the diversity of coliform genera typical for HTST-pasteurized fluid milk in the Northeast US.

Five Enterobacteriaceae Genera Represent the Majority of Coliform Bacteria Isolated from HTST-Pasteurized Fluid Milk Samples

The most commonly isolated genera from the pasteurized fluid milk samples examined in this study were *Enterobacter* (42% of total isolates [100/240]), *Hafnia* (13% of total isolates [31/240]), *Citrobacter* (12% of total isolates [28/240]), *Serratia* (10% of total isolates [24/240]), and *Raoultella* (9% of total isolates [22/240]) (Table 2.1). Combined, these genera accounted for

85% of the total coliforms isolated from the pasteurized fluid milk samples. These five most common genera were also found across processing plants with both *Citrobacter* and *Enterobacter* isolated from samples collected from 12 of the 21 processing plants that had coliform positive samples (Table 2.1). Isolation of different genera over shelf-life did not show any apparent patterns; for example, *Enterobacter* was consistently the most commonly isolated genus across days 7, 10, 14, 17, and 21, representing between 45 and 63% of isolates obtained on the different days of shelf-life (Table 2.2).

The diversity of coliform genera isolated from samples representing a given plant also showed considerable range; 6 different genera were found in 3/21 plants, 5 different genera were found in 2/21 plants, 4 different genera were found in 1/21 plants, 3 different genera were found in 6/21 plants, 2 different genera were found in 6/21 plants, and only 1 genus was found in 3/21 processing plants. Among the plants included here, samples were typically collected over 2 or sometimes 3 visits to a given plant with 4 to 6 months between visits. Interestingly, for 10 of the study plants, isolates representing the same genus were obtained from samples collected over separate visits (Table 2.3). For three plants (plants A, F, and S; see Table 2.3), isolates with the same 16SrDNA-ST were obtained from samples collected over two separate visits, providing preliminary evidence for a persistent coliform source in these facilities. Overall, characterization to 16SrDNA-ST allowed for considerable discrimination among the 240 non-redundant isolates, as supported by an SID of 0.85.

Table 2.2. Frequency of common coliform genera throughout fluid milk shelf life[1]

Genus	No. of isolates (%)[3] obtained on						Total no. of isolates
	Initial day	Day 7[2]	Day 10[2]	Day 14[2]	Day 17[2]	Day 21[2]	
Enterobacter	2 (100%)	15 (45%)	26 (45%)	33 (45%)	12 (60%)	12 (63%)	**100**
Hafnia	0 (0%)	6 (18%)	10 (17%)	14 (19%)	1 (5%)	0 (0%)	**31**
Citrobacter	0 (0%)	5 (15%)	9 (16%)	11 (15%)	2 (10%)	1 (5%)	**28**
Serratia	0 (0%)	6 (18%)	4 (7%)	8 (11%)	3 (15%)	3 (16%)	**24**
Raoultella	0 (0%)	1 (3%)	9 (16%)	7 (10%)	2 (10%)	3 (16%)	**22**
Total (n)	**2**	**33**	**58**	**73**	**20**	**19**	**205**

[1] Common genera were defined as those genera represented by 5 or more non-redundant isolates (Non-redundant isolates were defined as isolates that did not share the same plant, date, sample, and genus as any other isolate

[2] Days are +/- 1 day

[3] Percent values are calculated to represent the % of isolates within a given day that represent the different genera; % values may not add up to 100% due to rounding.

Table 2.3. Plants where the same coliform genera and 16SrDNA-ST were isolated from HTST-pasteurized fluid milk samples collected on separate sampling visits.

Plant	Re-isolated genus	16SrDNA-ST [1](no. of isolates) for isolates representing the re-isolated genus		
		visit A	visit B	visit C
A	*Enterobacter*	40 (3), 43 (1), 49 (2)	40 (2), 58 (1)	n/a
B	*Enterobacter*	40 (5)	44 (1), 52 (8), 53 (1), 56 (1), 57 (1)	n/a
E	*Cedecea*	13 (2)	12 (4)	n/a
F	*Raoultella*	81 (4)	80 (3), 81 (4)	n/a
H	*Citrobacter*	38 (2)	14 (1), 15 (2), 16 (1)	n/a
O	*Raoultella*	80 (6)	83 (12), 84 (1)	n/a
P	*Buttiauxella*	10 (1)	4 (1), 9 (1), 5 (1)	n/a
Q	*Citrobacter*	17 (1)	0	21 (1), 26 (1)
	Enterobacter	0	40 (51), 43 (4), 46 (1)	54 (2)
	Serratia	95 (1), 97 (1)	89 (17), 90 (1), 91 (1), 92 (1)	0
S	*Enterobacter*	40 (6)	40 (2), 43 (1)	n/a
T	*Enterobacter*	49 (11), 48 (3)	40 (4)	n/a

[1]16SrDNA-ST = 16SrDNA sequence type; two isolates are defined as having the same 16SrDNA-ST if they share an identical partial 16S rDNA sequence; n/a = no visit conducted.

All Coliform Isolates Tested Displayed a 2 Log Increase in Bacterial Growth over 10 Days at 6°C

Across the 104 representative coliform isolates chosen for cold growth analysis at 6°C for 10 d (Table 2.4), the average bacterial count at days 0, 2, 4, 6, 8, and 10 was 2.08 ± 0.57 mean log_{10}cfu/mL, 2.76 ± 0.86 mean log_{10}cfu/mL, 4.11 ± 1.66 mean log_{10}cfu/mL, 5.70 ± 2.29 mean log_{10}cfu/mL, 7.66 ± 2.15 mean log_{10}cfu/mL, and 8.23 ± 1.92 mean log_{10}cfu/mL, respectively. Based on the development of bacterial numbers over 10 d at 6°C, we calculated, for each bacterial isolate, the parameter "bacterial growth at 6°C over 10 d", which was defined as log bacterial numbers at day 10 minus log bacterial numbers at day 0. Average bacterial growth of all coliform isolates tested at 6°C was 5.88 ± 1.62 logs. Of the 104 representative isolates tested, 74 isolates had growth greater than 5 logs over 10 d at 6°C; the remaining isolates all showed between 2 and 5 logs of growth. The most substantial average growth at 6°C was observed for isolates representing the genera *Rahnella*, *Serratia*, and *Buttiauxella* (7.98 logs, 7.31 logs, and 6.86 logs, respectively; Table 2.4). The average bacterial growth over 10 d at 6°C of each of the five most commonly isolated genera (*Citrobacter*, *Enterobacter*, *Hafnia*, *Raoultella*, and *Serratia*) exceeded 5.10 logs. Comparisons of the growth over 10 d at 6°C for different isolates within the five most common genera showed considerable diversity of growth within certain genera (Figure 2.1). Isolates within the genus *Citrobacter* had the largest range of bacterial growth, spanning over 5 logs from 2.00 to 7.13 logs (Figure 2.1). *Serratia* and *Enterobacter* isolates showed log growth at 6°C ranging from 4.64 to 8.24 logs and 2.77 to 6.99 logs, respectively. *Raoultella* and *Hafnia* isolates showed the smallest range of bacterial growth, ranging from 4.00 to 6.51 logs and 5.07 to 7.60 logs, respectively (Figure 2.1).

Table 2.4. Phenotypic characteristics of 104 representative coliform isolates tested for cold growth as well as lipolytic and proteolytic capabilities.

Genus	Number of isolates tested[1]	Δ growth [log d10 - log d0)]	D14 Lipolysis Score[2]			D14 Proteolysis Score[3]		
			(-)	(+)	(++)	(-)	(+)	(++)
Citrobacter	27	5.22	22	5	0	4	22	1
Enterobacter	20	5.12	6	14	0	7	13	0
Serratia	17	7.31	0	1	16	4	6	7
Buttiauxella	10	6.86	10	0	0	0	8	2
Hafnia	9	6.57	9	0	0	7	2	0
Kluyvera	5	5.18	4	1	0	2	3	0
Pantoea	5	6.57	1	4	0	2	3	0
Raoultella	5	5.17	4	1	0	3	2	0
Rahnella	3	7.98	3	0	0	0	3	0
Cedecea	2	3.47	0	2	0	0	2	0
Leclercia	1	2.87	0	1	0	1	0	0
Total	104	-	59	29	16	30	64	10

[1]16S rDNA sequence data were used to select isolates for phenotypic characterization (cold growth, proteolytic and lipolytic capabilities)

[2] Lipolysis was tested on Spirit Blue Agar; (-) = no visible clearing around streak area; (+) = small zone of clearing around streak area (≤ 2mm) but remainder of the plate remains blue; (++) = clearing around the streak area greater than 2 mm

[3] Proteolysis was tested on Skim Milk Agar): (-) = no visible clearing around streak area; (+) = noticeable clearing around streak area; (++) = obvious clear zone and opaque zone surrounding the streak area

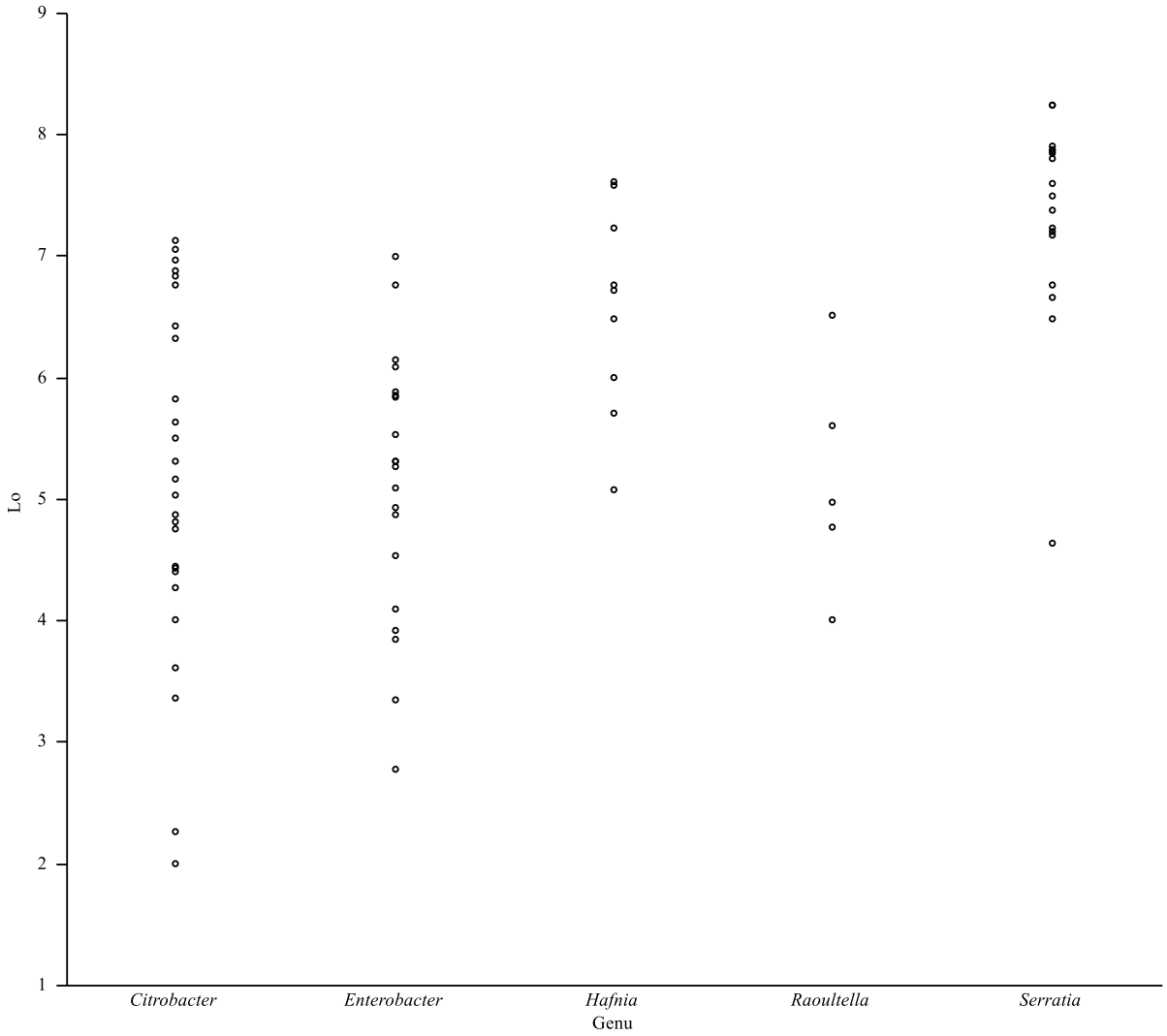

Figure 2.1. Log growth from day 0 to day 10 of refrigerated storage at 6°C for each isolate within commonly isolated genera from pasteurized fluid milk. (Log growth = log bacterial numbers at day 10 – log bacterial numbers at day 0)

Coliform Bacteria Isolated from HTST Pasteurized Fluid Milk Samples differ in Lipolysis and Proteolysis Phenotypes

The 104 representative isolates tested for cold growth were also analyzed for lipolytic and proteolytic activity after incubation at 6°C for 14 d. Among the 104 isolates tested for lipolysis, 59 isolates scored (-) [no visible clearing around streak area on SB], 29 isolates scored (+) [small zone of clearing (≤ 2 mm) around streak area on SB], and 16 isolates scored (++) [clearing > 2 mm on SB] (Table 2.4). There was a significant association between the lipolytic activity [with isolates defined as showing lipolytic activity if they were scored as a (+) or (++)] and genus of the isolates tested ($P < 0.0001$; Fisher's exact test), indicating that the proportion of isolates that show lipolytic capabilities differs between coliform genera. Only *Serratia* isolates showed strong lipolysis (++), with the majority of the representative *Serratia* (93%) isolates characterized showing this lipolysis phenotype. For both the genus *Enterobacter* and the genus *Pantoea* the majority of representative isolates (70% and 80%) scored as lipolytic (+) on SB; for all other genera the majority of isolates showed no evidence for lipolysis (-).

Among the 104 representative isolates tested for proteolysis, 30 isolates scored (-) [no visible clearing around the streak area on SMA], 64 isolates scored as (+) [noticeable clearing around the streak area on SMA], and 10 isolates scored as (++) [an obvious clear zone and opaque zone surrounding the streak area on SMA] (Table 2.4). Similar to the analysis of lipolytic activity, there was a significant association between the proteolytic activity [defined as a (+) or (++)] and genera of the isolates tested ($P = 0.01$; Fisher's exact test), indicating that the proportion of isolates that show proteolytic capabilities differs between coliform genera. The genera *Serratia* and *Buttiauxella* had the largest percentage of representative isolates score as (++), 41% and 20% of isolates, respectively.

DISCUSSION

While detection of coliforms is frequently used as an indicator of the hygienic quality of HTST-pasteurized fluid milk, our understanding of the taxonomic and phenotypic diversity of coliforms associated with this product produced in contemporary processing plants is limited. This study showed that a considerable diversity of coliforms can be found in fluid milk and also defined some of the most common coliform genera found in HTST-pasteurized fluid milk. Coliforms isolated from HTST-pasteurized milk in general showed considerable growth capabilities at 6°C and virtually all coliforms isolated here should be considered "psychrotolerant" (defined here as having the ability to grow at refrigeration temperatures). Overall, our data suggest that the PPC population of coliform bacteria is comprised of diverse members capable of cold growth as well as lipolysis and proteolysis. Thus, these bacteria can likely lead to loss of product quality due to spoilage and sensory defects.

Coliform Bacteria Isolated from HTST-Pasteurized Milk Samples are Diverse

Eleven different coliform bacteria genera were represented by the isolates obtained from HTST-pasteurized fluid milk samples in the Northeast US. These data suggest that post-pasteurization contaminants from fluid milk represent a diverse collection of coliform bacteria rather than a homogenous population. In previous work that characterized Gram-negative bacteria obtained from bulk tank raw milk samples stored at 4°C, researchers isolated coliform bacteria representing the genera *Citrobacter*, *Enterobacter*, *Hafnia*, and *Serratia* from samples collected in the US in 1997 (Jayarao and Wang, 1999) and representing the same genera (*Citrobacter*, *Enterobacter*, *Hafnia*, and *Serratia*) from samples collected in Ireland in 2005 (Jayarao BM, and L. Wang, 1999; Kagkli et al., 2007). These genera match 4 of the 5 most commonly isolated coliform genera reported here. When specifically assessing pasteurized milk

and dairy products collected in 1988 in South Africa, researchers isolated coliform bacteria representing the genera *Citrobacter*, *Enterobacter*, *Serratia*, *Hafnia*, and *Cedecea* (Wessels et al., 1989), also consistent with our results reported here. Hence, similar coliform genera appear to be consistently isolated from dairy associated samples collected in different studies and in different countries. Interestingly, Säde and colleagues isolated *Hafnia* spp., *Pantoea* spp., *Rahnella* spp., *Buttiauxella* spp., and *Serratia* spp. from modified atmosphere packed meats and poultry collected in Finland at the end of chilled storage at 6°C (Säde et al., 2013), suggesting that similar psychrotolerant coliforms are present in different types of food products.

Three coliform genera (*Raoultella*, *Leclercia*, and *Kluyvera*) were isolated here but had not been reported in the key studies on coliforms in dairy products discussed above (Wessels et al., 1989; Jayarao and Wang, 1999; Kagkli et al., 2007). The genus *Raoultella* was previously classified as part of the genus *Klebsiella* (a coliform group commonly found associated with dairy plant equipment) (Drancourt et al., 2001; Tang et al., 2009; Zadoks et al., 2011). *Raoultella* spp. may thus have been reported as *Klebsiella* in previous studies; interestingly none of the isolates obtained here were identified as *Klebsiella*. Conversely, the isolation of *Leclercia* spp. and *Kluyvera* spp. were unexpected due to their limited association with food products thus far in previous research. The genus *Leclercia* was formerly known as part of the genus *Escherichia* and has been documented as having associations with clinical human infections (Temesgen et al., 1997; Hess et al., 2008). Little is known about the ecology and diversity of the genus *Kluyvera*, which was formerly identified as enteric group 8, except that it is a coliform bacteria associated with the environment as well as clinical human infections (Farmer et al., 1981; Sarria et al., 2001; Carter and Evans, 2005). Importantly though, overall only 2 and 13 of the 240 non-redundant coliform isolates characterized here represented the genera *Leclercia* and

Kluyvera. Additionally, the *Leclercia* isolates were all obtained from a single visit at one dairy plant and therefore may be the result of a factor unique to this plant.

Coliform Isolates Obtained from Pasteurized Fluid Milk Have the Ability to Not Only Survive but to Grow at 6°C

Previous research has suggested that coliform bacteria are poor competitors at low temperatures (Fuquay et al., 2011). However, in the absence of competition with other bacteria for nutrients, coliform bacteria have been found to grow at a wide range of temperatures, including refrigeration temperatures (Stead, 1986; Fuquay et al., 2011). Therefore, pasteurized fluid milk kept at refrigeration temperature may be an ideal environment for these spoilage organisms, particularly if the milk is not contaminated with any other psychrotolerant bacteria. The ability of coliforms to grow in commercial pasteurized milk is also supported by a few previous studies, although limited data are available on the specific Gram-negative organisms that caused fluid milk spoilage, except for a few studies on psychrotolerant *Pseudomonas* spp., which are often considered key organisms linked to fluid milk spoilage (Schröder et al., 1982; Eneroth et al., 2000; Dogan and Boor, 2003; Martin et al., 2012). For example, a recent 10-year study of HTST-pasteurized fluid milk quality in NYS found that 11 % (111/1008) of fluid milk samples showed coliform counts of more than 10 cfu/mL prior to day 14 of refrigerated storage (Martin et al., 2012). Additionally, a 1982 study of PPC of fluid milk in 5 United Kingdom dairies (Schröder et al., 1982) also found that 100% of isolates from spoiled milk (showing off-flavor or bitterness as early as at 10 d of storage at 5°C) represented Gram-negative rods (Schröder et al., 1982). Additionally, a 1999 study of PPC of fluid milk in both a Swedish and a Norwegian dairy found close to 20% of all pasteurized milk samples positive for Gram-negative psychrotolerant bacteria by 11 d of storage at 7°C (Eneroth et al, 2000). While isolates from the

two previous studies (Schröder et al., 1982 ; Eneroth et al, 2000) were not further characterized and thus may include non-coliform Gram-negatives (e.g., *Pseudomonas* spp.), these data further support the importance of Gram-negative spoilage organisms in HTST-pasteurized fluid milk.

Our data also showed that a large proportion of the coliforms characterized showed considerable growth capabilities at 6°C, with 71% of isolates showing > 5 log growth over 10 d. This is consistent with previous data, which showed that that the majority of coliform genera isolated from milk and dairy products in South Africa were capable of growth on SPC agar incubated at 7°C for 10 d (Wessels et al., 1989); specifically, 100% of isolates representing *Citrobacter freundii* (13), *Enterobacter agglomerans* (10), *Serratia marcescens* (3), and *Hafnia alvei* (3) grew on SPC agar after 10 d at 7°C (Wessels et al., 1989). Our data also showed that some coliform genera included isolates that differed considerably in their ability to grow at 6°C in SMB. This may represent overall phenotypic diversity within a genus or may reflect distinct characteristics for different species within a given genus. For example, in a previous study, Wessels and colleagues reported that 100% of *Enterobacter agglomerans* and 89% of *Enterobacter cloacae* isolates were capable of psychrotrophic growth (Wessels et al., 1989).

Post-Pasteurization Contamination Coliform Isolates Obtained from HTST-Pasteurized Fluid Milk Vary in Lipolytic and Proteolytic Capabilities

As sensory defects in fluid milk resulting from lipolysis and proteolysis of the product have direct impact on both overall milk quality as well as consumer acceptance (Ma et al., 2000; Santos et al., 2003), we further characterized representative coliform isolates obtained here for proteolytic and lipolytic capabilities when grown at 6°C. The release of free fatty acids from triglycerides in the milk, known as lipolysis, results in a 'rancid' flavor defect of the product (Shipe et al., 1978). The accumulation of small peptides as a result of proteolysis of milk results

in a flavor defect that is described as ‘bitter’ or ‘astringent’ (Harwalkar et al., 1989; Ma et al., 2000). Our results showed the isolates representing 11 different coliform genera identified had varying capabilities of lipolysis; 57% of the isolates showed no lipolytic reaction, while 43% showed lipolysis, scoring (+) or (++). This observation differs from previous research (Juven et al., 1981; Wessels et al., 1989; Plou et al., 1998), which indicated limited lipolytic capabilities of coliform bacteria isolated from dairy products and raw milk in Israel and South Africa. For example, one study showed that among 75 Enterobacteriaceae isolates from dairy products tested for lipolysis, only 7 isolates (9%) (3/17 *Enterobacter cloacae*; 2/3 *Serratia marcescens*; 1/14 *Klebsiella oxytoca*; 1/2 *K. pneumonia*) showed lipolytic activity on Victoria Blue Butterfat agar (Wessels et al., 1989). These differences in the frequency of lipolysis phenotype among dairy isolates from different studies may at least be partially due to different methods of measuring lipolysis or may be due to small isolate sets characterized in some of these previous studies. For example, in one study examining the changes in refrigerated milk caused by psychrotolerant strains of Enterobacteriaceae isolated from raw milk, researchers found that none of the 6 strains produced any lipolytic activity on Tween 80 agar (Juven et al., 1981). Our study reported here not only tested > 15 times more isolates, allowing for better representation of strain diversity, but also used Spirit Blue agar for lipolytic activity detection. This is relevant as previous work has suggested that the lipolytic activity detected with either Tween 80 or Tributyrin as the substrate is not correlated (Plou et al., 1998). This is important as both Tributyrin and Polysorbate (Tween) 80 are the substrates in the Spirit Blue agar lipase reagent, which was used here. We surmise lipolysis activity determined with the Spirit Blue agar lipase reagent is more relevant to dairy products than lipolysis activity detected with only Tween 80, as the reagent contains both

Tributyrin (butyric acid is a component of milk fat) and Tween 80 (containing oleic acid, another component of milk fat) (Garton, 1963; MacGibbon and Taylor, 2006).

Similar to the lipolysis results, we found that proteolytic activity differed between and within the coliform genera in the current study; nearly 30% of the isolates tested showed no observed proteolysis, while only 10% of the isolates, predominantly representing the genus *Citrobacter*, showed a strong proteolytic reaction. By comparison, previous studies (Juven et al., 1981; Wessels et al., 1989) reported limited proteolytic capabilities for Enterobacteriaceae bacteria isolated from dairy products. For example, one study (Juven et al., 1981) reported that none of six psychrotolerant Enterobacteriaceae isolates obtained from raw milk (collected in Israel) exhibited proteolytic activity when evaluated on Skim Milk agar. Similarly, Wessels and colleagues reported that Enterobacteriaceae strains isolated from milk and dairy products (collected in South Africa) were generally non-proteolytic (Wessels et al., 1989); the only species identified as proteolytic in this study were *E. cloacae*, *K. oxytoca*, and *S. rubidaea*. Overall, our results suggest that proteolytic activity may be more common among dairy associated coliforms than reported in previous studies.

CONCLUSIONS

Our data indicate that psychrotolerant coliforms introduced as PPC in fluid milk represent considerable taxonomic diversity. In addition, a considerable proportion of these coliform isolates were found to be characterized by the ability to exhibit lipolytic and proteolytic activity. As contamination with psychrotolerant coliforms has been shown to influence the sensory characteristics and consumer acceptability of fluid milk (Fairbairn and Law, 1986; Martin et al., 2012), continued efforts are clearly needed to reduce PPC with coliforms. Future efforts are

needed to more specifically define the sources of coliform PPC, particularly since our data suggested that some coliform types (either specific genera or 16SrDNA-STs) were repeatedly isolated in a given facility, suggesting point source introduction of coliforms in some plants. Additionally, future work is needed to explore possible differences in cold growth and enzymatic activity capabilities within multiple coliform genera and subtypes. Overall, general hygienic issues within a fluid milk processing plant may lead to not only the introduction of coliform bacteria, but to the potential PPC with a diverse group of coliform contaminants capable of having a direct impact on pasteurized milk quality and the consumer's sensory experience.

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Supplemental Table 2.1. Coliform isolates (402) isolated from HTST-pasteurized fluid milk, genus of isolate, 16srDNA-ST of isolate determined by partial 16S sequence, enzymatic testing scores of representative isolates (104), bacterial counts and overall growth of chosen non-redundant isolates.

Isolate	Genus	16srDNA-ST	Plant	Day 14 Proteolysis Score[1]	Day 14 Lipolysis Score[2]	Day 0 Count (log)	Day 10 Count (log)	Δ Growth [D10-D0 count] (log)	Representative Isolate
A5-0001	*Hafnia*	59	D	-	-	2.20	9.43	7.23	X
A5-0002	*Hafnia*	59	D	n/a	n/a	n/a	n/a	n/a	
A5-0003	*Citrobacter*	14	H	+	-	2.58	9.40	6.82	X
A5-0004	*Serratia*	85	C	++	++	2.66	9.82	7.16	X
A5-0005	*Serratia*	93	C	++	++	2.26	10.1	7.84	X
A5-0006	*Hafnia*	63	D	n/a	n/a	n/a	n/a	n/a	
A5-0007	*Hafnia*	63	D	n/a	n/a	n/a	n/a	n/a	
A5-0008	*Citrobacter*	15	H	+	-	2.20	8.52	6.32	X
A5-0009	*Citrobacter*	15	H	n/a	n/a	n/a	n/a	n/a	
A5-0010	*Serratia*	87	C	++	++	2.61	10.48	7.87	X
A5-0011	*Serratia*	88	C	++	++	2.36	10.26	7.90	X
A5-0012	*Pantoea*	72	H	-	+	1.48	9.88	8.40	X
A5-0013	*Pantoea*	73	H	-	+	2.90	9.45	6.55	X
A5-0014	*Hafnia*	63	D	-	-	2.51	10.08	7.57	X
A5-0015	*Citrobacter*	16	H	+	-	1.48	8.45	6.97	X
A5-0016	*Serratia*	85	C	n/a	n/a	n/a	n/a	n/a	
A5-0018	*Hafnia*	63	D	n/a	n/a	n/a	n/a	n/a	
A5-0019	*Hafnia*	63	D	n/a	n/a	n/a	n/a	n/a	
A5-0020	*Rahnella*	78	H	+	-	1.00	9.90	8.90	X
A5-0021	*Serratia*	93	H	n/a	n/a	n/a	n/a	n/a	
A5-0022	*Serratia*	85	H	n/a	n/a	n/a	n/a	n/a	
A5-0023	*Serratia*	87	H	n/a	n/a	n/a	n/a	n/a	
J3-0001	*Citrobacter*	19	U	+	-	2.08	9.12	7.04	X
J3-0002	*Kluyvera*	67	U	-	-	2.15	9.55	7.40	X
J3-0003	*Citrobacter*	25	U	+	-	1.70	8.58	6.88	X
J3-0004	*Citrobacter*	23	U	+	-	2.04	8.81	6.77	X
J3-0005	*Citrobacter*	24	U	+	-	1.85	8.89	7.04	X
J3-0006	*Citrobacter*	20	U	+	-	1.70	8.83	7.13	X
J3-0007	*Buttiauxella*	4	P	+	-	1.00	9.97	8.97	X
J3-0008	*Buttiauxella*	9	P	+	-	1.30	9.86	8.56	X
J3-0009	*Buttiauxella*	5	P	+	-	1.85	8.52	6.67	X
J3-0010	*Enterobacter*	53	B	+	+	1.79	5.71	3.92	X
J3-0011	*Enterobacter*	44	B	+	+	1.79	5.87	4.08	X
J3-0012	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0013	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0014	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0015	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0016	*Enterobacter*	52	B	+	+	2.12	5.96	3.84	X
J3-0017	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0018	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0019	*Enterobacter*	52	B	n/a	n/a	n/a	n/a	n/a	
J3-0020	*Citrobacter*	21	Q	+	-	2.09	6.53	4.44	X
J3-0021	*Citrobacter*	26	Q	+	-	1.91	6.72	4.81	X
J3-0022	*Enterobacter*	54	Q	n/a	n/a	n/a	n/a	n/a	
J3-0023	*Enterobacter*	54	Q	-	+	2.08	7.00	4.92	X
J3-0024	*Enterobacter*	55	M	n/a	n/a	n/a	n/a	n/a	
J3-0026	*Enterobacter*	43	M	n/a	n/a	n/a	n/a	n/a	
J3-0027	*Enterobacter*	40	M	n/a	n/a	n/a	n/a	n/a	
J3-0028	*Enterobacter*	56	B	-	+	2.00	8.15	6.15	X
J3-0029	*Enterobacter*	57	B	-	+	2.00	7.30	5.30	X
J3-0030	*Enterobacter*	40	M	n/a	n/a	n/a	n/a	n/a	
J3-0031	*Citrobacter*	39	M	+	-	2.05	6.93	4.88	X
J3-0032	*Citrobacter*	39	M	n/a	n/a	n/a	n/a	n/a	
J3-0033	*Raoultella*	84	O	n/a	n/a	n/a	n/a	n/a	
J3-0034	*Raoultella*	84	O	n/a	n/a	n/a	n/a	n/a	
J3-0037	*Raoultella*	84	O	n/a	n/a	n/a	n/a	n/a	
J3-0038	*Raoultella*	84	O	+	-	1.70	7.30	5.60	X

J3-0039	*Raoultella*	84	O	n/a	n/a	n/a	n/a	n/a	
J3-0040	*Raoultella*	84	O	n/a	n/a	n/a	n/a	n/a	
J3-0041	*Leclercia*	71	A	n/a	n/a	n/a	n/a	n/a	
J3-0042	*Leclercia*	71	A	-	+	1.01	3.88	2.87	X
J3-0043	*Enterobacter*	40	A	n/a	n/a	n/a	n/a	n/a	
J3-0044	*Enterobacter*	40	A	n/a	n/a	n/a	n/a	n/a	
J3-0045	*Citrobacter*	33	T	n/a	n/a	n/a	n/a	n/a	
J3-0046	*Citrobacter*	34	T	n/a	n/a	n/a	n/a	n/a	
J3-0047	*Citrobacter*	34	T	-	-	2.15	7.78	5.63	X
J3-0048	*Citrobacter*	33	T	+	-	1.85	7.00	5.15	X
J3-0049	*Hafnia*	61	T	-	-	1.95	8.66	6.71	X
J3-0050	*Hafnia*	61	T	n/a	n/a	n/a	n/a	n/a	
J3-0051	*Enterobacter*	40	T	n/a	n/a	n/a	n/a	n/a	
J3-0052	*Enterobacter*	40	T	n/a	n/a	n/a	n/a	n/a	
J3-0053	*Enterobacter*	40	T	n/a	n/a	n/a	n/a	n/a	
J3-0054	*Enterobacter*	40	T	n/a	n/a	n/a	n/a	n/a	
J3-0055	*Cedecea*	11	E	n/a	n/a	n/a	n/a	n/a	
J3-0056	*Cedecea*	11	E	n/a	n/a	n/a	n/a	n/a	
J3-0057	*Cedecea*	11	E	+	+	1.91	5.55	3.64	X
J3-0058	*Cedecea*	11	E	n/a	n/a	n/a	n/a	n/a	
J3-0059	*Rahnella*	77	E	+	-	1.70	9.22	7.52	X
J3-0060	*Rahnella*	77	E	n/a	n/a	n/a	n/a	n/a	
J3-0061	*Raoultella*	83	O	n/a	n/a	n/a	n/a	n/a	
J3-0065	*Raoultella*	83	O	n/a	n/a	n/a	n/a	n/a	
J3-0066	*Raoultella*	83	O	n/a	n/a	n/a	n/a	n/a	
J3-0067	*Raoultella*	83	O	n/a	n/a	n/a	n/a	n/a	
J3-0068	*Raoultella*	83	O	n/a	n/a	n/a	n/a	n/a	
J3-0071	*Raoultella*	83	O	n/a	n/a	n/a	n/a	n/a	
J3-0072	*Raoultella*	84	O	+	-	1.95	6.93	4.98	X
J3-0075	*Enterobacter*	58	A	-	-	3.25	7.78	4.53	X
J3-0077	*Citrobacter*	22	F	n/a	n/a	n/a	n/a	n/a	
J3-0078	*Citrobacter*	22	F	-	-	2.73	7.00	4.27	X
J3-0079	*Citrobacter*	22	F	n/a	n/a	n/a	n/a	n/a	
J3-0080	*Citrobacter*	22	F	n/a	n/a	n/a	n/a	n/a	
J3-0082	*Raoultella*	81	F	n/a	n/a	n/a	n/a	n/a	
J3-0083	*Kluyvera*	66	F	+	+	1.00	4.38	3.38	X
J3-0084	*Citrobacter*	37	F	+	+	1.71	6.47	4.76	X
J3-0089	*Raoultella*	80	F	n/a	n/a	n/a	n/a	n/a	
J3-0090	*Raoultella*	80	F	n/a	n/a	n/a	n/a	n/a	
J3-0094	*Raoultella*	80	F	n/a	n/a	n/a	n/a	n/a	
J3-0095	*Raoultella*	81	L	n/a	n/a	n/a	n/a	n/a	
J3-0096	*Raoultella*	81	L	n/a	n/a	n/a	n/a	n/a	
J3-0097	*Raoultella*	82	L	-	+	1.60	5.60	4.00	X
J3-0098	*Raoultella*	82	L	n/a	n/a	n/a	n/a	n/a	
J3-0099	*Enterobacter*	40	L	-	+	2.48	8.00	5.52	X
J3-0100	*Enterobacter*	40	L	n/a	n/a	n/a	n/a	n/a	
J3-0101	*Enterobacter*	50	C	n/a	n/a	n/a	n/a	n/a	
J3-0102	*Enterobacter*	51	C	+	-	1.94	6.80	4.86	X
J3-0103	*Enterobacter*	50	C	n/a	n/a	n/a	n/a	n/a	
J3-0104	*Enterobacter*	41	C	+	+	1.91	7.17	5.26	X
J3-0105	*Enterobacter*	50	C	-	-	2.23	5.00	2.77	X
J3-0106	*Enterobacter*	55	C	-	-	1.95	5.30	3.35	X
J3-0107	*Enterobacter*	50	C	n/a	n/a	n/a	n/a	n/a	
J3-0108	*Enterobacter*	50	C	n/a	n/a	n/a	n/a	n/a	
P4-0743	*Kluyvera*	68	N	-	-	2.00	5.00	3.00	X
P4-0745	*Citrobacter*	31	N	-	-	3.70	5.95	2.25	X
P4-0746	*Citrobacter*	28	N	-	-	1.85	6.28	4.43	X
P4-0747	*Hafnia*	65	N	n/a	n/a	n/a	n/a	n/a	
P4-0748	*Raoultella*	80	N	n/a	n/a	n/a	n/a	n/a	
P4-0749	*Hafnia*	65	N	n/a	n/a	n/a	n/a	n/a	
P4-0750	*Hafnia*	65	N	n/a	n/a	n/a	n/a	n/a	
P4-0751	*Hafnia*	65	N	n/a	n/a	n/a	n/a	n/a	
P4-0752	*Hafnia*	65	N	-	-	2.18	8.18	6.00	X
P4-0753	*Hafnia*	65	N	n/a	n/a	n/a	n/a	n/a	
P4-0754	*Hafnia*	60	N	n/a	n/a	n/a	n/a	n/a	
P4-0755	*Hafnia*	62	G	n/a	n/a	n/a	n/a	n/a	
P4-0756	*Hafnia*	62	G	n/a	n/a	n/a	n/a	n/a	
P4-0757	*Hafnia*	62	G	n/a	n/a	n/a	n/a	n/a	
P4-0758	*Hafnia*	62	G	-	-	2.23	7.30	5.07	X
P4-0759	*Pantoea*	74	G	n/a	n/a	n/a	n/a	n/a	

P4-0760	*Pantoea*	74	G	n/a	n/a	n/a	n/a	n/a	
P4-0761	*Pantoea*	74	G	n/a	n/a	n/a	n/a	n/a	
P4-0762	*Pantoea*	74	G	+	+	2.04	7.00	4.96	X
P4-0763	*Hafnia*	60	G	n/a	n/a	n/a	n/a	n/a	
P4-0764	*Hafnia*	60	G	n/a	n/a	n/a	n/a	n/a	
P4-0765	*Hafnia*	60	G	n/a	n/a	n/a	n/a	n/a	
P4-0766	*Hafnia*	60	G	-	-	2.30	8.00	5.70	X
P4-0767	*Pantoea*	76	G	n/a	n/a	n/a	n/a	n/a	
P4-0768	*Pantoea*	76	G	n/a	n/a	n/a	n/a	n/a	
P4-0769	*Pantoea*	76	G	+	+	1.70	7.30	5.60	X
P4-0770	*Pantoea*	74	G	n/a	n/a	n/a	n/a	n/a	
P4-0771	*Serratia*	86	G	n/a	n/a	n/a	n/a	n/a	
P4-0772	*Serratia*	86	G	+	+	1.95	8.61	6.66	X
P4-0773	*Serratia*	86	G	n/a	n/a	n/a	n/a	n/a	
P4-0774	*Serratia*	86	G	n/a	n/a	n/a	n/a	n/a	
P4-0775	*Citrobacter*	38	H	n/a	n/a	n/a	n/a	n/a	
P4-0777	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0778	*Enterobacter*	47	H	+	+	1.85	7.70	5.85	X
P4-0779	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0780	*Enterobacter*	42	H	+	+	2.11	8.20	6.09	X
P4-0781	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0782	*Enterobacter*	43	H	n/a	n/a	n/a	n/a	n/a	
P4-0783	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0784	*Citrobacter*	38	H	+	+	1.91	5.26	3.35	X
P4-0785	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0786	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0787	*Enterobacter*	40	H	n/a	n/a	n/a	n/a	n/a	
P4-0789	*Enterobacter*	43	H	n/a	n/a	n/a	n/a	n/a	
P4-0799	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0800	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0801	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0802	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0803	*Hafnia*	65	P	n/a	n/a	n/a	n/a	n/a	
P4-0804	*Hafnia*	65	P	n/a	n/a	n/a	n/a	n/a	
P4-0805	*Hafnia*	65	P	n/a	n/a	n/a	n/a	n/a	
P4-0806	*Hafnia*	65	P	n/a	n/a	n/a	n/a	n/a	
P4-0807	*Serratia*	93	P	n/a	n/a	n/a	n/a	n/a	
P4-0808	*Hafnia*	64	P	+	-	1.48	9.08	7.60	X
P4-0809	*Buttiauxella*	10	P	+	-	1.00	7.60	6.60	X
P4-0810	*Hafnia*	60	P	n/a	n/a	n/a	n/a	n/a	
P4-0811	*Serratia*	89	Q	+	++	1.31	9.17	7.86	X
P4-0812	*Serratia*	90	Q	+	++	1.70	9.19	7.49	X
P4-0813	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0814	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0815	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0816	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0817	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0818	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0819	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0820	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0821	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0822	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0827	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0828	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0829	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0830	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0831	*Enterobacter*	40	P	n/a	n/a	n/a	n/a	n/a	
P4-0832	*Rahnella*	79	P	n/a	n/a	n/a	n/a	n/a	
P4-0833	*Hafnia*	64	P	n/a	n/a	n/a	n/a	n/a	
P4-0834	*Hafnia*	65	P	n/a	n/a	n/a	n/a	n/a	
P4-0835	*Hafnia*	65	P	n/a	n/a	n/a	n/a	n/a	
P4-0836	*Serratia*	93	P	n/a	n/a	n/a	n/a	n/a	
P4-0837	*Hafnia*	60	P	n/a	n/a	n/a	n/a	n/a	
P4-0838	*Hafnia*	62	P	n/a	n/a	n/a	n/a	n/a	
P4-0839	*Hafnia*	62	P	n/a	n/a	n/a	n/a	n/a	
P4-0840	*Serratia*	89	Q	-	++	1.90	9.14	7.24	X
P4-0841	*Serratia*	91	Q	-	++	1.70	8.90	7.20	X
P4-0842	*Serratia*	89	Q	+	++	1.31	9.10	7.79	X
P4-0843	*Serratia*	89	Q	-	++	1.49	9.08	7.59	X
P4-0844	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	

P4-0845	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0846	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0847	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0848	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0849	*Kluyvera*	70	M	+	-	2.40	8.29	5.89	X
P4-0850	*Kluyvera*	69	M	+	-	2.11	8.35	6.24	X
P4-0851	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0852	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0853	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0854	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0855	*Kluyvera*	69	M	n/a	n/a	n/a	n/a	n/a	
P4-0856	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0857	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0858	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0859	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0860	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0861	*Enterobacter*	43	Q	n/a	n/a	n/a	n/a	n/a	
P4-0862	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0863	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0864	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
P4-0865	*Enterobacter*	43	S	n/a	n/a	n/a	n/a	n/a	
P4-0866	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
P4-0867	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
P4-0868	*Serratia*	89	Q	+	++	1.01	9.24	8.23	X
P4-0869	*Serratia*	92	Q	-	++	1.00	9.24	8.24	X
P4-0870	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0871	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0872	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0873	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0874	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0875	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0876	*Buttiauxella*	1	M	+	-	1.00	8.48	7.48	X
P4-0877	*Buttiauxella*	1	M	n/a	n/a	n/a	n/a	n/a	
P4-0878	*Buttiauxella*	1	M	n/a	n/a	n/a	n/a	n/a	
P4-0879	*Rahnella*	79	M	+	-	1.00	8.52	7.52	X
P4-0880	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0881	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0882	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0883	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0884	*Enterobacter*	46	Q	+	-	1.70	8.60	6.90	X
P4-0885	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0886	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0887	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0888	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0889	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0890	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0891	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0892	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0893	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0894	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0895	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0896	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0897	*Enterobacter*	43	Q	n/a	n/a	n/a	n/a	n/a	
P4-0898	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0899	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0900	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0901	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0902	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0903	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0904	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0905	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0906	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0907	*Serratia*	89	Q	n/a	n/a	n/a	n/a	n/a	
P4-0908	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0909	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0910	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0911	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0912	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0913	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0914	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	

P4-0915	*Enterobacter*	43	Q	n/a	n/a	n/a	n/a	n/a	
P4-0916	*Enterobacter*	43	Q	n/a	n/a	n/a	n/a	n/a	
P4-0917	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0918	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0919	*Enterobacter*	40	Q	n/a	n/a	n/a	n/a	n/a	
P4-0920	*Buttiauxella*	3	E	++	-	1.00	8.60	7.60	X
P4-0921	*Buttiauxella*	6	E	+	-	3.25	8.64	5.39	X
P4-0922	*Buttiauxella*	7	E	n/a	n/a	n/a	n/a	n/a	
P4-0923	*Buttiauxella*	7	E	++	-	1.91	8.50	6.59	X
P4-0927	*Buttiauxella*	8	E	+	-	3.29	8.64	5.35	X
P4-0930	*Buttiauxella*	7	E	n/a	n/a	n/a	n/a	n/a	
P4-0932	*Enterobacter*	43	A	n/a	n/a	n/a	n/a	n/a	
P4-0933	*Enterobacter*	40	A	n/a	n/a	n/a	n/a	n/a	
P4-0934	*Enterobacter*	40	A	n/a	n/a	n/a	n/a	n/a	
P4-0935	*Enterobacter*	40	A	n/a	n/a	n/a	n/a	n/a	
P4-0936	*Citrobacter*	27	A	n/a	n/a	n/a	n/a	n/a	
P4-0937	*Citrobacter*	27	A	n/a	n/a	n/a	n/a	n/a	
P4-0939	*Citrobacter*	27	A	+	+	1.90	6.94	5.04	X
P4-0940	*Enterobacter*	49	A	+	+	1.70	6.79	5.09	X
P4-0941	*Enterobacter*	49	A	n/a	n/a	n/a	n/a	n/a	
P4-0942	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0943	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0948	*Citrobacter*	27	A	n/a	n/a	n/a	n/a	n/a	
P4-0949	*Citrobacter*	27	A	n/a	n/a	n/a	n/a	n/a	
P4-0950	*Citrobacter*	27	A	n/a	n/a	n/a	n/a	n/a	
P4-0951	*Citrobacter*	27	A	n/a	n/a	n/a	n/a	n/a	
P4-0952	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0953	*Enterobacter*	48	T	n/a	n/a	n/a	n/a	n/a	
P4-0954	*Enterobacter*	48	T	+	+	1.00	6.88	5.88	X
P4-0955	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0956	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0957	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0958	*Enterobacter*	48	T	n/a	n/a	n/a	n/a	n/a	
P4-0959	*Enterobacter*	49	T	+	+	1.00	6.31	5.31	X
P4-0960	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0961	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0962	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0963	*Enterobacter*	49	T	n/a	n/a	n/a	n/a	n/a	
P4-0964	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0965	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0966	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0967	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0968	*Serratia*	94	R	++	++	2.36	8.85	6.49	X
P4-0969	*Serratia*	96	R	+	++	1.95	8.72	6.77	X
P4-0970	*Serratia*	95	R	++	++	1.60	8.98	7.38	X
P4-0971	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0972	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0973	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0974	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0975	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0976	*Raoultella*	81	F	n/a	n/a	n/a	n/a	n/a	
P4-0977	*Raoultella*	81	F	+	-	1.00	5.77	4.77	X
P4-0978	*Raoultella*	81	F	n/a	n/a	n/a	n/a	n/a	
P4-0979	*Raoultella*	81	F	n/a	n/a	n/a	n/a	n/a	
P4-0980	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0981	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0982	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0983	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0984	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0985	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0986	*Enterobacter*	43	R	n/a	n/a	n/a	n/a	n/a	
P4-0987	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0988	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0989	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0990	*Enterobacter*	43	R	n/a	n/a	n/a	n/a	n/a	
P4-0991	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0992	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0993	*Enterobacter*	40	R	n/a	n/a	n/a	n/a	n/a	
P4-0994	*Enterobacter*	43	R	n/a	n/a	n/a	n/a	n/a	

W4-0241	*Enterobacter*	45	S	n/a	n/a	n/a	n/a	n/a	
W4-0242	*Enterobacter*	45	B	+	-	1.00	7.76	6.76	X
W4-0243	*Enterobacter*	45	B	n/a	n/a	n/a	n/a	n/a	
W4-0244	*Serratia*	95	Q	n/a	n/a	n/a	n/a	n/a	
W4-0245	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
W4-0246	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
W4-0247	*Enterobacter*	40	B	n/a	n/a	n/a	n/a	n/a	
W4-0248	*Kluyvera*	69	Q	n/a	n/a	n/a	n/a	n/a	
W4-0249	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
W4-0250	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
W4-0251	*Enterobacter*	40	B	n/a	n/a	n/a	n/a	n/a	
W4-0252	*Enterobacter*	40	B	n/a	n/a	n/a	n/a	n/a	
W4-0253	*Raoultella*	80	Q	n/a	n/a	n/a	n/a	n/a	
W4-0254	*Pantoea*	75	Q	+	-	1.30	8.64	7.34	X
W4-0255	*Serratia*	97	Q	++	++	2.36	7.00	4.64	X
W4-0256	*Citrobacter*	30	S	+	-	1.70	5.30	3.60	X
W4-0257	*Enterobacter*	40	S	n/a	n/a	n/a	n/a	n/a	
W4-0258	*Citrobacter*	17	Q	+	-	1.30	5.30	4.00	X
W4-0259	*Buttiauxella*	2	Q	+	-	2.08	7.50	5.42	X
W4-0260	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0261	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0262	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0263	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0264	*Hafnia*	65	E	+	-	2.48	8.96	6.48	X
W4-0265	*Hafnia*	65	E	n/a	n/a	n/a	n/a	n/a	
W4-0266	*Raoultella*	80	O	n/a	n/a	n/a	n/a	n/a	
W4-0267	*Raoultella*	80	O	n/a	n/a	n/a	n/a	n/a	
W4-0268	*Hafnia*	65	E	n/a	n/a	n/a	n/a	n/a	
W4-0269	*Hafnia*	65	E	n/a	n/a	n/a	n/a	n/a	
W4-0270	*Hafnia*	83	E	n/a	n/a	n/a	n/a	n/a	
W4-0271	*Hafnia*	83	E	-	-	2.18	8.94	6.76	X
W4-0272	*Raoultella*	80	E	n/a	n/a	n/a	n/a	n/a	
W4-0273	*Raoultella*	80	E	n/a	n/a	n/a	n/a	n/a	
W4-0274	*Raoultella*	80	E	n/a	n/a	n/a	n/a	n/a	
W4-0275	*Raoultella*	80	E	-	-	2.51	9.01	6.50	X

W4-0276	*Citrobacter*	24	E	n/a	n/a	n/a	n/a	n/a	
W4-0277	*Citrobacter*	18	E	+	-	1.85	3.85	2.00	X
W4-0278	*Raoultella*	80	O	n/a	n/a	n/a	n/a	n/a	
W4-0279	*Raoultella*	80	O	n/a	n/a	n/a	n/a	n/a	
W4-0280	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0281	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0282	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0283	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0284	*Cedecea*	12	E	n/a	n/a	n/a	n/a	n/a	
W4-0285	*Cedecea*	12	E	+	+	1.31	4.61	3.30	X
W4-0286	*Raoultella*	80	E	n/a	n/a	n/a	n/a	n/a	
W4-0287	*Hafnia*	65	E	n/a	n/a	n/a	n/a	n/a	
W4-0288	*Hafnia*	62	E	n/a	n/a	n/a	n/a	n/a	
W4-0289	*Citrobacter*	29	E	++	-	2.26	8.69	6.43	X
W4-0290	*Raoultella*	80	O	n/a	n/a	n/a	n/a	n/a	
W4-0291	*Raoultella*	80	O	n/a	n/a	n/a	n/a	n/a	
W7-2250	*Citrobacter*	32	K	n/a	n/a	n/a	n/a	n/a	
W7-2251	*Citrobacter*	35	K	+	+	1.30	7.12	5.82	X
W7-2252	*Citrobacter*	32	K	n/a	n/a	n/a	n/a	n/a	
W7-2253	*Citrobacter*	32	K	+	+	1.30	5.70	4.40	X
W7-2254	*Citrobacter*	13	J	-	-	1.00	6.49	5.49	X
W7-2255	*Citrobacter*	36	J	-	-	1.00	6.30	5.30	X
W7-2256	*Citrobacter*	32	K	n/a	n/a	n/a	n/a	n/a	
W7-2257	*Citrobacter*	32	K	n/a	n/a	n/a	n/a	n/a	
W7-2258	*Enterobacter*	40	J	n/a	n/a	n/a	n/a	n/a	
W7-2259	*Enterobacter*	40	J	n/a	n/a	n/a	n/a	n/a	
W7-2260	*Cedecea*	12	I	n/a	n/a	n/a	n/a	n/a	
W7-2261	*Enterobacter*	43	J	+	+	2.28	8.11	5.83	X
W7-2262	*Enterobacter*	40	J	n/a	n/a	n/a	n/a	n/a	
W7-2263	*Enterobacter*	40	J	n/a	n/a	n/a	n/a	n/a	
W7-2264	*Enterobacter*	43	J	n/a	n/a	n/a	n/a	n/a	

[1] Proteolysis scores (Skim Milk Agar): (-) = no visible clearing around the streak area; (+) = noticeable clearing around the streak area; (++) = an obvious clear zone and opaque zone surrounding the streak area

[2]Lipolysis scores (Spirit Blue Agar): (-) = no visible clearing around the streak area; (+) = small zone of clearing around the streak area (≤ 2 mm) but remainder of the plate remains blue; (++) = clearing around the streak area greater than 2 mm

CHAPTER 3

IDENTIFICATION OF DAIRY FARM MANAGEMENT PRACTICES ASSOCIATED WITH THE PRESENCE OF PSYCHROTOLERANT SPOREFORMERS IN BULK TANK MILK

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ABSTRACT

Some strains of sporeforming bacteria, e.g., *Bacillus* spp. and *Paenibacillus* spp., can survive pasteurization and subsequently grow at refrigeration temperatures, causing pasteurized fluid milk spoilage. To identify farm management practices associated with different levels of sporeformers in raw milk, a bulk tank sample was obtained from and a management and herd health questionnaire was administered to 99 New York State dairy farms. Milk samples were spore pasteurized (SP) (80ºC (176ºF), 12 min) and subsequently analyzed for most probable number and for sporeformer counts on initial day of SP, and after refrigerated storage (6ºC) at 7, 14, and 21 d post-SP. Management practices were analyzed for association with sporeformer counts and bulk tank somatic cell counts, respectively. Sixty-two farms had high sporeformer growth (≥ 3 log cfu/mL at any day post-SP) with an average sporeformer count of 5.20 ± 1.41 mean $\log_{10}$ cfu/mL at 21 d post-SP. Thirty-seven farms had low sporeformer numbers (< 3 log cfu/mL for all days post-SP) with an average sporeformer count of 0.75 ± 0.94 mean $\log_{10}$ cfu/mL at 21d post-SP. Farms with > 25% of cows with dirty udders in the milking parlor were 3.15 times more likely to be in the high category than farms with ≤ 10% of milking cows with dirty udders. Farms with < 200 cows were 3.61 times more likely to be in the high category than farms with ≥ 200 cows. Management practices significantly associated with increased bulk tank somatic cell count were ‘a lack of use of the California Mastitis Test at freshening’ and ‘> 25%

of cows with dirty udders observed in the milking parlor'. Changes in management practices associated with cow cleanliness may directly ensure longer shelf-life and higher quality pasteurized fluid milk.

(**Key words**: *Bacillus* spp., *Paenibacillus* spp., spoilage, management practice)

INTRODUCTION

Food loss due to microbial spoilage is costly for the United States (Buzby and Hyman, 2012). Of the > 5 billion gallons of pasteurized fluid milk meant for consumption in the U.S. every year, one-fifth is discarded by consumers and foodservice businesses (IDFA, 2010; Gunders, 2012). Bacterial spoilage is the predominant limiting factor in the shelf-life of pasteurized fluid milk (Boor, 2001; Durak et al., 2006). Microbes can be present in pasteurized milk through two different routes: (i) survival of pasteurization by bacteria present in raw milk, and (ii) post-pasteurization contamination (PPC) of the product. Microbes associated with the former route of spoilage are generally Gram-positive sporeformers (Boor and Murphy, 2002; Huck et al., 2007a).

In general, in the absence of PPC, sporeforming bacteria are the predominant residual organisms in pasteurized fluid milk. Gram-positive *Bacillus* spp. and *Paenibacillus* spp. form heat-resistant spores that can withstand high temperature short time (HTST) pasteurization commonly used for fluid milk processing (Collins, 1981; Fromm and Boor, 2004; Ranieri et al., 2009). The ability of these organisms to survive heat treatment and of certain strains to grow at refrigerated storage temperatures results in milk spoilage (Washam et al., 1977; Huck et al., 2008). Whereas *Bacillus* spp. are usually the predominant genera present up to 7 d post-pasteurization in milk held at 6^{o}C, *Paenibacillus* spp. often dominate later in shelf-life (i.e. at 17 d and beyond) (Fromm and Boor, 2004; Ranieri et al., 2009). The metabolic activities of these sporeforming spoilage bacteria can lead to loss of product quality, including curdling and off odors or flavors (Ageitos et al., 2007; Dutt et al., 2009). Reduction or elimination of these bacterial contaminants can result in extension of fluid milk shelf-life, which would advance the dairy industry by providing overall higher product quality.

Eliminating sporeforming bacteria is challenging, as these organisms are ubiquitously found in the environment, including in soil, on plant surfaces, in decaying matter, and in mammalian digestive tracts (Gilliam et al., 1984; Gilliam, 1985; Sarkar, 1991; Fredrickson and Onstott, 1996; Nicholson, 2002). Sporeforming bacteria have been isolated from the dairy farm environment. For example, *Bacillus sporothermodurans* was isolated from feed concentrates on 17 Belgian dairy farms (Scheldeman et al., 2002). *Bacillus* spp. also were prevalent on Scottish dairy farms, with *B. licheniformis* most commonly isolated from the dairy farm environment (excluding grass and soil samples) and *B. cereus*, *B. circulans*, *B. firmis*, *B. licheniformis*, *B. subtilis*, *B. coagulans*, *B. sphaericus*, and *B. mycoides* isolated from raw bulk tank/silo milk (Crielly et al., 1994). *Paenibacillus* spp. have been isolated from silage, dairy cow feed concentrate, and raw milk (Vaerewijick et al., 2001; te Giffel et al., 2002; Scheldeman et al., 2004). Sporeformers have been isolated along the dairy product processing continuum, from milk trucks to packaged final products, with plant factors such as processing parameters, including pasteurization temperatures (Ranieri et al., 2009; Martin et al., 2011), significantly affecting microbiological quality of the final pasteurized product. Identification of the same bacterial subtypes in both raw and pasteurized milk samples suggests that pasteurized fluid milk spoilage can result from bacteria that enter raw milk on the farm (Huck et al., 2007b).

We hypothesized that identifying specific farm practices that associate with different sporeformer levels could allow development of specific, actionable recommendations for production of raw bulk tank milk with low sporeformer numbers. Therefore, the objectives of this study were to: (i) assess the prevalence and diversity of psychrotolerant sporeformers isolated from bulk tank milk; and (ii) evaluate possible associations between on-farm management practices and levels of psychrotolerant sporeformers in bulk tank milk.

MATERIALS AND METHODS

Herd selection

A cross-sectional observational study was conducted on 99 New York State (**NYS**) dairy farms from May 2009 to June 2010. Herds were selected from the Quality Milk Production Services (QMPS) (College of Veterinary Medicine, Cornell University, Ithaca, NY) program clientele at four different QMPS locations, representing different regions in NYS, including Ithaca (14 herds), Canton (29 herds), Cobleskill (47 herds), and Geneseo (9 herds). Farms were selected based on willingness to participate; participating farms represented a range of herd sizes and historical bulk tank somatic cell counts (BtSCC) as detailed in the 'Results' section. All participants were fully informed of the design of the study, the nature of the data being collected and its future use, and were aware that the study was voluntary. Each participant signed an informed consent document acknowledging the above items.

Survey design

The survey used in this study was adapted from an existing QMPS survey that included questions on herd health, housing cleanliness, equipment maintenance, milking time procedures, and medication usage. The revised survey was a one-page document that included all of these topics except medication usage. 'Percent dirty udders in the milking area' was evaluated using the University of Wisconsin Udder Hygiene Scoring Chart (Dairy Team Extension, 2002). The factor was defined as the percentage of cows whose rear legs and rear udder area were moderately covered with dirt (10-30% of surface area) or covered with caked on dirt (> 30% of surface area). The questions on the survey were either open-ended or close-ended with binary (Y/N) answers. The survey was designed to be administered orally to the participant. This

survey was pre-tested for ease of understanding on QMPS staff who would be administering the survey as well as on the first five farms included in the study.

Survey administration & bulk tank sampling

QMPS technicians were trained on survey administration by a supervisor. Each location had a single, designated, and trained technician who administered the survey and collected samples. Technicians administering the surveys were trained to obtain objective answers without being leading, to focus on quantitative/numeric answers, and to follow training guides for any necessary subjective scores or observations. Surveys were administered during the same farm visit as when bulk tank samples were obtained.

Bulk tank milk at each farm was sampled using two sterile dip vials and one National Dairy Herd Information Association (DHIA) vial, which were immediately stored on ice packs in a cooler and held at $\leq 6^{o}C$. Raw bulk tank milk samples (250 mL in each of two vials) were shipped overnight to the laboratory in Styrofoam coolers packed with ice packs. Temperature data recorders were included in each shipment and the temperature of the sample was recorded immediately upon arrival to the Milk Quality Improvement Program (MQIP) laboratory (Department of Food Science, Cornell University, Ithaca, NY). Any samples with temperatures > 6°C during transit or upon arrival were rejected and the farm was re-sampled. DHIA vials were shipped directly from QMPS locations to Dairy One (Ithaca, NY) for BtSCC analysis using a Fossomatic FC ESCC automated SCC reader (Foss Inc., Hillerød, Denmark).

Microbiological evaluation of milk samples

For each farm, the two sample vials (250 mL each) were commingled into one sterile 500 mL glass bottle. Raw milk samples were inverted completely 25 times prior to removal of an

aliquot for microbiological analyses which included; (i) total bacteria count on Standard Plate Count (SPC) agar (Difco, BD Diagnostics, Franklin Lakes, NJ) as described by Laird et al. (2004); (ii) Psychrotrophic Bacteria Count (PBC) (Laird et al., 2004) and (iii) Preliminary Incubation (PI) count (Martin et al., 2011).

The remaining raw milk was distributed equally among three sterile 250 mL glass bottles for spore pasteurization (SP), performed by heat treating each of the three bottles (~150 mL each) at 80°C (176°F) for 12 min, followed by immediate cooling on ice. After cooling to 6°C, the samples in each bottle were commingled into a sterile 500 mL glass bottle. The bottle was fully inverted 25 times and 200 µL samples were spiral plated onto duplicate SPC agar plates. The remaining SP milk samples were then split equally into three sterile 250 mL glass bottles and held at 6°C for microbiological testing (SPC) at 7, 14 and 21 d post-SP. Additionally, a modified five tube most probable number (MPN) method (Davidson et al., 2004) was used to enumerate very low numbers of psychrotolerant sporeformers not achievable by plating techniques (Supplemental Figure 3.1). The MPN method was performed on SP samples as follows: 10 mL of SP milk was aliquoted into each of five sterile screw capped tubes, 1 mL of SP milk was aliquoted into each of five sterile screw capped tubes containing 9 mL of sterile skim milk broth (SMB) (1:10 dilution) and finally, 0.1 mL of SP milk was aliquoted into each of five sterile screw capped tubes containing 9.9 mL of sterile SMB (1:100 dilution). Each of the fifteen tubes was vortexed and then incubated at 6°C for 21 d prior to spiral plating on SPC. Plates were evaluated for presence or absence of growth. MPN data were interpreted into quantitative results using a five tube MPN table (Davidson et al., 2004).

Bacterial isolate collection

Bacterial colonies representing visually distinct morphologies (typically 1 to 4 colonies per sample) were selected and streaked for purity on brain heart infusion (BHI) agar (Difco, Franklin Lakes, NJ) from SPC agar plates used for bacterial enumeration on each sampling date. Pure cultures were grown overnight in BHI broth at 32ºC prior to freezing in 15% glycerol at -80ºC. A total of 1,182 isolates were collected over the duration of the study. Isolate information can be found at www.foodmicrobetracker.com.

Molecular characterization & identification of isolates

Isolates selected for molecular characterization were obtained from SP milk samples plated on day Initial (DI), day 7 (D7), day 14 (D14), and day 21 (D21). Isolates collected from MPN plates were not characterized. The methods described by Huck et al. (2007a) were used to determine species identification for psychrotolerant sporeformer isolates. Briefly, cultures were streaked for colony isolation from frozen stock onto BHI agar and grown at 32ºC for 24 h. A sterile toothpick was used to sample an isolated colony and PCR was performed to amplify the 632 bp *rpoB* gene fragment (Huck et al., 2007a). After verifying amplification by gel electrophoresis, DNA fragments were purified using the ExoSap method (Dugan et al., 2002) and bidirectional sequencing with PCR primers was performed by the Life Sciences Core Laboratory Center (Cornell University, Ithaca, NY) using Sanger sequencing. Genus and/or species assignment were obtained using 16S rDNA for isolates not identifiable with *rpoB* sequencing. Sequence alignment and allelic type (AT) assignment methods were as described by Ivy et al. (2012).

Prediction of cold growth analysis of isolates

To identify isolates likely to be able to grow at refrigeration temperatures and hence, to cause fluid milk spoilage, isolates obtained in this study were compared phylogenetically with sporeforming bacterial isolates that had been tested previously for cold growth ability (defined as growth over 24 d at 6°C) (Ivy et al., 2012). Briefly, *rpoB* ATs from 444 isolates in this study were compared with all *rpoB* ATs analyzed by Ivy et al. (2012) in a parsimony phylogenetic tree that was constructed using PAUP (version 4, Sinauer Associates Inc., Sunderland, MA). Isolates were selected to reduce duplication, i.e., isolates from duplicate plates of the same milk sample with the same AT were eliminated to reduce overrepresentation of a given AT. Study isolates were considered to be members of previously described clades if they grouped closely with one or more of the earlier analyzed ATs (Ivy et al., 2012).

Statistical analyses

All statistical analyses were performed in SAS (version 9.3, SAS Institute Inc., Carey, NC). The distributions of sporeformer counts at each day of refrigerated storage were plotted. Individual farm sample results were separated into two categories based on the distributions of sporeformer numbers during the entire 21 d storage period at 6ºC; a 'low' category indicates that sporeformer counts remained < 3 log cfu/mL during the entire storage period and a 'high' category indicates that sporeformer counts were ≥ 3 log cfu/mL at any day during the storage period. Sporeformer count data and raw milk test data were log transformed prior to analyses. A correlation matrix analysis was performed between raw milk microbiological test results (raw milk SPC, PBC, and PI), MPN test results, and sporeformer counts at each day of refrigerated storage (DI, D7, D14, and D21) to explore possible correlations using the restricted maximum likelihood method and calculation of R^2 values.

Logistic regression analysis modeling for the 'high' category data was used to assess associations between management practices and sporeformer categories. Both bivariate and multivariate models were investigated. Survey response categories were evaluated for possible correlations; if present, the more biologically relevant factor (determined by a closer relationship to the milking parlor area or milking practices) was chosen for analysis. Additionally, factor levels were assessed for potential collapse into fewer levels due to low distributions for different levels within a single factor. Variables having a significant ($P \leq 0.10$) association in a bivariate logistic regression model with the 'high' sporeformer category were included in a multivariate logistic regression model. Any nonsignificant ($P > 0.10$) variables were removed from the multivariate model using backward elimination (starting with the least significant factor). For BtSCC, SCC data were log transformed and analyzed using a linear regression model. Variables with significant ($P \leq 0.10$) associations with the 'high' sporeformer category in a bivariate linear regression model were subsequently included in a multivariate linear regression model. Variables with non-significant ($P > 0.10$) associations were removed from the multivariate model using backward elimination. A one-way ANOVA was used to identify differences between 'low' and 'high' categories ($P \leq 0.05$) for each raw milk microbiological test and each day of refrigerated storage. A one-way ANOVA was also used to identify the distribution of MPN over the factor levels within the management practices 'percent dirty udders in the milking area' and 'herd size' ($P \leq 0.05$). Chi-square tests were performed on the distribution of genera between the high and low category and between days of refrigerated storage ($P \leq 0.05$) and a Fisher's exact test was performed on the distribution of clades classified as cold growing and non-cold growing between the high and low categories ($P \leq 0.05$).

RESULTS

Milk from diverse farms shows two distinct microbial growth patterns following spore pasteurization and subsequent incubation at 6°C

Herd information collected included herd size, cow breed, number of milking cows, number of milkings per day, housing type, average milk production, and BtSCC. Herd sizes ranged from 15 to 3,100 cows, with a mean size of 265 ± 484 cows. Cows were housed in tiestalls (57%), freestalls (15%), stanchions (13%), or on pasture (15%). Cow breeds included Holstein (51%), Jersey (4%), and multiple or mixed breeds (45%). The number of lactating cows per farm ranged from 10 to 2,800 (mean of 279 ± 472 lactating cows), with farms milking between 1 and 3 times daily (5% at 1x, 75% at 2x, and 20% at 3x). Average milk production across farms was 8,308 kg (18,317 lbs) ± 3,282 kg (7,237 lbs) per cow per year and ranged from 1,564 kg (3,450 lbs) to 12,727 kg (28,060 lbs).

After milk collected from the 99 participating farms was treated by SP and then incubated at 6°C, milk from 37 farms (37%) showed limited or no bacterial growth (< 3 log cfu/mL at each test day through D21 post-SP); these farms will be referred to as 'low category' farms. Milk from the other 62 farms (63%) showed considerable bacterial growth after SP, reaching bacterial numbers of ≥ 3 log cfu/mL during 21 d post-SP (Figure 3.1); these farms will be referred to as 'high category' farms. Milk from 48/62 farms (77%) in the 'high category' reached bacterial numbers of ≥ 20,000 cfu/mL on D21 post-SP and would not meet the standard of < 20,000 cfu/mL for Grade A pasteurized milk set by the Pasteurized Milk Ordinance (PMO) (FDA, 2011).

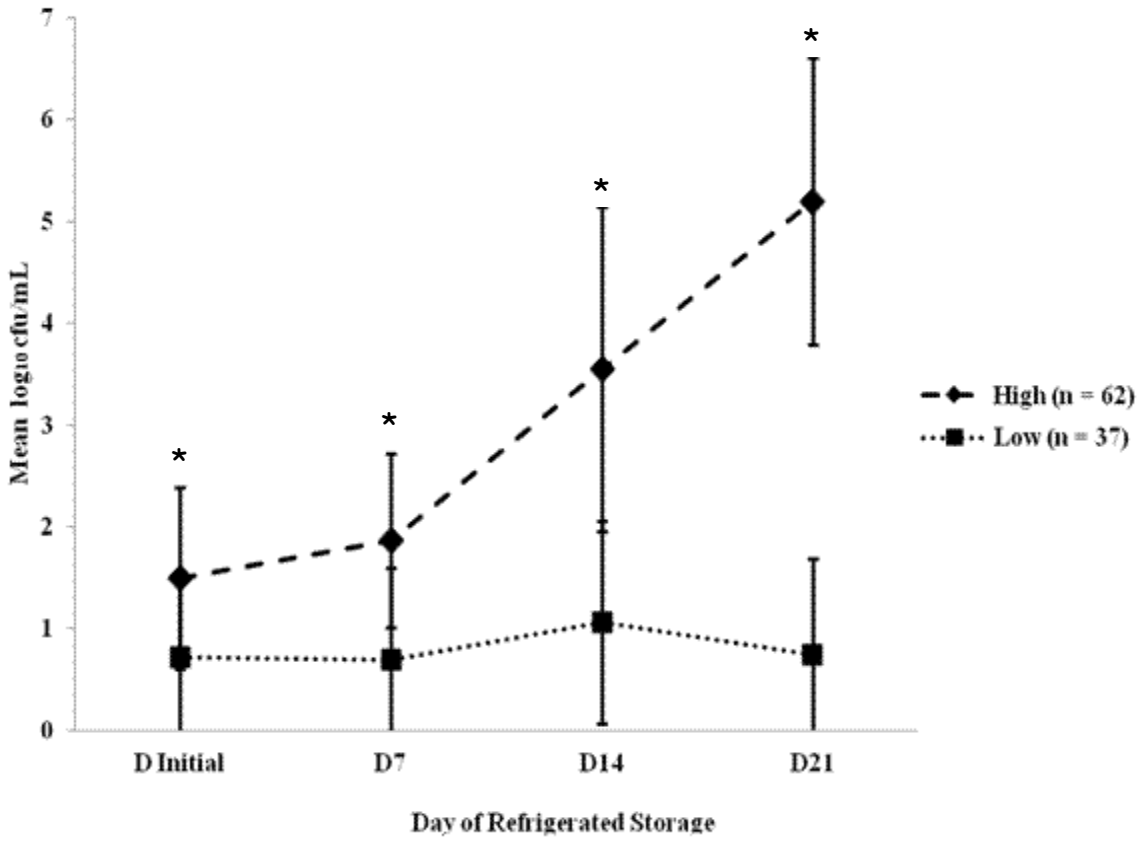

Figure 3.1. Sporeformer bacteria levels for spore pasteurized (SP) (80°C (176°F), 12 min) milk from high and low category farms over 21 days post-SP at 6°C refrigerated storage. Data represent mean $\log_{10}$cfu/mL and bars indicate mean ± SD for each day of refrigerated storage. Significance (*) at ($P \leq 0.001$).

At each day of refrigerated storage, bacterial numbers for the high and low category farms (Figure 3.1) differed significantly ($P \leq 0.001$). The mean bacterial counts at D21 post-SP for samples from farms in the low and high categories were 0.75 ± 0.94 and 5.20 ± 1.41 $\log_{10}$ cfu/mL, respectively. Bacterial counts in the spore treated samples will be referred to as 'sporeformer counts' in all subsequent sections as (i) the SP treatment should kill vegetative cells and (ii) isolates obtained were classified into sporeformer genera (see below).

Raw milk yielding SP-treated milk samples that classified into the low and high categories differed in selected microbiological raw milk parameters

The BtSCC for the 99 milk samples evaluated ranged from 56,000 to 2,062,000 cells/mL; mean BtSCC for milk from the low and high category farms (256,000 and 368,000 cells/mL, respectively) were not significantly different (Table 3.1). Raw milk SPC ranged from 1.86 to 6.75 $\log_{10}$ cfu/mL; mean raw milk SPC values for milk from the low and high category farms (3.68 and 3.94 mean $\log_{10}$ cfu/mL, respectively) also were not significantly different. Raw milk PI counts ranged from 2.30 to 7.11 $\log_{10}$ cfu/mL; mean raw milk PI counts for milk from the low and high category farms (4.75 and 5.39 $\log_{10}$ cfu/mL, respectively) were significantly different ($P = 0.0045$). PBC values for the raw milk samples ranged from 1.00 to 6.93 $\log_{10}$ cfu/mL; mean PBC for milk from the low and high category farms (2.41 and 3.11 $\log_{10}$ cfu/mL) were significantly different ($P = 0.0027$; Table 3.1). MPN for psychrotolerant sporeformers (determined on SP-treated milk incubated at 6°C) ranged from < 0.01 to > 24 MPN/mL; mean MPN for milk from the low and high category farms (0.11 and 2.13 MPN/mL) were significantly different ($P = 0.0153$; Table 3.1).

Table 3.1. Mean bacterial counts for raw milk microbiological quality analyses and bulk tank SCC for farms categorized with low or high sporeformer milk

Microbiological Analysis[1]	Low Category[2] (n)	High Category[3] (n)
BtSCC (mean cells/mL ± SD) (61)	256,000 ± 370,000[a] (37)	368,000 ± 168,000 [a]
Raw SPC (mean $\log_{10}$ cfu/mL ± SD)	3.68 ± 0.83[a] (37)	3.94 ± 0.83[a] (62)
PI (mean $\log_{10}$ cfu/mL ± SD)	4.75 ± 0.93[a] (36)	5.39 ± 1.10[b] (59)
PBC (mean $\log_{10}$ cfu/mL ± SD)	2.41 ± 0.82[a] (31)	3.11 ± 1.13[b] (60)
MPN (mean MPN/mL ± SD)	0.11 ± 0.14[a] (35)	2.13 ± 4.82[b] (59)

[1] BtSCC = bulk tank SCC; Raw SPC = standard plate count of raw milk; PI = preliminary incubation count of raw milk; PBC = psychrotrophic bacteria count of raw milk; MPN = most probable number count in bulk tank samples.
[2] Low category defined as bacterial counts < 3 log cfu/mL for all days post- spore pasteurization (SP).
[3] High category defined as bacterial counts ≥ 3 log cfu/mL at any day post-SP.
[4] Different superscript letters (a, b) between low and high categories indicates a significant difference ($P \leq 0.05$) in means.

MPN data were available for 94/99 of the bulk tank samples. Overall, 10/35 samples categorized as low at D21 post-SP showed no growth in any of the MPN tubes (MPN < 0.01), whereas only 3/59 samples in the high category showed MPN < 0.01 (different at $P = 0.0014$; Fisher's exact test). Further analysis showed significant ($P < 0.05$) correlation between each of the raw milk tests used (raw milk SPC, PI, PBC, MPN, and BtSCC) and D21 sporeformer counts for all samples, including those from both low and high category farms. These findings indicate that multiple microbiological tests provide insight into the overall microbiological quality of raw milk, i.e., poor quality milk generally performs poorly in multiple tests. R^2 values were very low, however, ranging from 0.25 to 0.38 for raw milk SPC, PI, PBC, MPN, and BtSCC, indicating that raw milk tests do not show a good correlation with D21 bacterial counts in SP-treated milk, i.e., results from the raw milk tests do not accurately predict D21 bacterial counts.

Bacillus and Paenibacillus are the predominant sporeformer genera isolated from milk samples that represent the low and the high categories

A total of 444 representative bacterial isolates obtained from the 99 milk samples at different time points after SP treatment (Table 3.2) were characterized by *rpoB* sequencing, which enabled classification to genus, species, and AT. Not surprisingly, substantially more isolates were available from the samples in the high category (374 isolates) versus the low category (70 isolates). Virtually all isolates characterized represented either *Bacillus* spp. (71.4%; 317/444 isolates) or *Paenibacillus* spp. (26.4%; 117/444 isolates) (Table 3.2). *Lysinibacillus* spp., *Planococcaceae* spp., *Psychrobacillus* spp., and *Virdibacillus arvi/arenosi* accounted for 2.2% (10/444) of the total isolates (Table 3.2). Genus distribution (*Bacillus* and *Paenibacillus*) did not differ significantly between isolates from samples in the low and high category ($P = 0.871$; Chi-square test).

Table 3.2. Numbers and prevalence of bacterial isolates obtained from spore-pasteurized (80ºC (176ºF), 12 min) bulk tank milk samples from high and low category farms at day Initial (DI), day 7 (D7), day 14 (D14), and day 21 (D21) of refrigerated storage at 6°C

Bacterial genus and species	High[1]					Low[2]						
	DI	D7	D14	D21	Total no. High	DI	D7	D14	D21	Total no. Low	Total no. of isolates	% of isolates
Bacillus spp. (total)	90	73	62	44	269	19	10	10	9	48	317	71.4
cereus	1	0	0	0	1	1	0	0	0	2	2	0.5
cereus s.l.	1	1	1	0	3	0	1	1	0	2	5	1.1
cf. aerophilus	6	3	0	0	9	0	1	0	0	1	10	2.3
cf. badius	0	0	1	0	1	0	0	0	0	0	1	0.2
cf. nealsonii	2	0	0	0	2	0	0	0	0	0	2	0.5
clausii	0	0	1	0	1	0	0	0	0	0	1	0.2
licheniformis	49	38	24	13	124	7	5	4	6	22	146	32.9
megaterium	0	1	0	0	1	2	0	0	1	3	4	0.9
muralis	0	0	0	0	0	0	0	1	0	1	1	0.2
pumilus	18	14	7	8	47	6	2	1	1	10	57	12.8
safensis	5	3	2	1	11	1	1	0	0	2	13	2.7
sp.	1	0	0	0	1	0	0	0	0	0	1	0.2
subtilis s.l.	7	4	6	3	20	2	0	0	0	2	22	5.0
weihenstephanensis	0	9	20	19	48	0	0	3	1	4	52	11.7
Paenibacillus spp. (total)	9	23	33	35	100	4	2	7	4	17	117	26.4
amylolyticus	0	0	0	1	1	0	0	0	0	0	1	0.2
amylolyticus s.l.	2	4	4	2	12	0	1	0	0	1	13	2.9
borealis	0	0	1	0	1	0	0	0	0	0	1	0.2
cf. *cookii*	3	1	0	0	4	1	0	1	0	2	6	1.4
cf. *pabuli*	0	1	1	1	3	1	0	0	0	1	4	0.9
cf. *peoriae*	1	4	11	10	26	2	0	2	1	5	31	7.0
graminis	0	1	2	6	9	0	0	1	2	3	12	2.7
lactis	1	0	0	0	1	0	0	0	0	0	1	0.2
macerans	2	0	0	0	2	0	0	0	0	0	2	0.5
odorifer	0	10	12	13	35	0	1	3	1	5	40	9.0
sp.	0	2	2	2	6	0	0	0	0	0	6	1.4
Lysinibacillus sp.	0	0	1	0	1	4	0	0	0	4	5	1.1
Planococcaceae sp.	0	0	0	0	0	0	1	0	0	1	1	0.2
Psychrobacillus sp.	1	0	0	0	1	0	0	0	0	0	1	0.2
Viridibacillus arvi/arenosi	0	1	1	1	3	0	0	0	0	0	3	0.7
TOTAL	100	97	97	80	374	27	13	17	13	70	444	100

[1] High category defined as bacterial count ≥ 3 log cfu/mL at any day post- spore pasteurization (SP) (80°C (176°F), 12 min).
[2] Low category defined as bacterial count < 3 log cfu/mL for all days post-SP.

Bacillus spp. represented 71.9% (269/374) and 68.6% (48/70) of the isolates characterized from the high and low category farms; *Paenibacillus* spp. represented 26.7% (100/374) and 24.3% (17/70) of isolates, respectively. The predominant *Bacillus* and *Paenibacillus* sp. among the characterized isolates were *B. licheniformis*, *B. pumilus*, and *B. weihenstephanensis* (Table 3.2) and *P. odorifer* and *P. cf. peoriae* (Table 3.2), respectively. Among all isolates obtained from milk at DI and D7 post-SP, 192 and 38 were classified as *Bacillus* and *Paenibacillus*, respectively; isolates obtained from milk at D14 and D21 represented 125 *Bacillus* and 79 *Paenibacillus* isolates. The proportion of *Paenibacillus* isolates was significantly higher among isolates from D14 and D21 (38% of all isolates collected at these two time points) as compared to isolates from DI and D7 (16% of all isolates collected at these two time points) ($P < 0.05$; Chi-square test).

rpoB sequence data also allowed for characterization of isolates to subtypes (*rpoB* AT); a total of 93 and 36 unique ATs were found among the characterized isolates representing high and low category samples, respectively. Importantly, *rpoB* AT data allowed us to cross reference the subtypes of the isolates characterized here to a reference collection of > 1,300 sporeformer isolates (Ivy et al., 2012). This previous study also classified the most prevalent clades ('families') of *rpoB* ATs as either having or not having the ability to grow at low temperatures (Table 3.3). Numerically, a higher proportion of isolates representing samples from the 'high category' farms represented clades classified as cold growing (55/80 isolates [69%]) as compared to isolates representing samples from the 'low category' farms (5/13 isolates [38%]) (Table 3.2); however, the proportions were not significantly different ($P = 0.06$; Fisher's exact test).

Table 3.3. Classification into previously defined cold growth clades of bacterial isolates obtained from spore-pasteurized (80°C (176°F), 12 min) milk samples at 21 days of refrigerated storage (6°C)

Sporeformer Clade[1]	Cold Growth Status[1, 2]	Number of D21 isolates within clade	
		High	Low
Bacillus licheniformis sensu lato	-	13	6
Bacillus megaterium	+/-	0	1
Bacillus pumilus	-	8	1
Bacillus safensis	-	1	0
Bacillus subtilis sensu lato	-	3	0
Bacillus weihenstephanensis	+	19	1
Viridibacillus sp.	+	1	0
Paenibacillus amylolyticus sensu lato	+	3	0
Paenibacillus graminis	+	6	2
Paenibacillus odorifer	+	13	1
Paenibacillus cf. *peoriae*	+	10	1
Paenibacillus sp.	+	3	0

[1] Cold Growth Clade definitions and Cold Growth Clade Status as described in Ivy et al. (2012).
[2] (+) = > 5.0 log cfu/mL growth over 21 days at 6°C; (+/-) = limited growth < 3.5 log cfu/ml over 21 days at 6°C; (-) = no growth over 21 days at 6°C.

Farms with a higher percentage of cows with dirty udders in the milking parlor are more likely to produce milk that represents the high category after SP treatment

Bivariate analyses of 47 farm management factors identified four factors that were significantly associated with the 'high' farm category ($P \leq 0.10$), including (i) the percent of cows with dirty udders observed in the milking area, (ii) use of treated water in hoses to spray down equipment, (iii) the percent of cows with dirty udders observed in the housing area, and (iv) the size of the current herd (Table 3.4). The risk factor 'percent dirty udders observed in the housing area' was not selected for inclusion in the subsequent multivariate analysis because it was highly correlated with percent dirty udders observed in the milking area ($R^2 = 0.9998$). After multivariate analysis, two factors were found to be significantly ($P < 0.10$) associated with the 'high' category farms, including (i) 'percent dirty udders in the milking area' and (ii) 'the size of the current herd' (Table 3.4). For the factor 'percent dirty udders in the milking area,' the final explanatory model indicated that, compared to a baseline of 0 to 10% dirty udders, farms with 11 to 25% dirty udders were 1.71 times more likely to be in the high category and farms with > 25% dirty udders were 3.15 times more likely to be in the high category (Table 3.5). For the factor 'herd size,' the final model indicated that, compared to a baseline of 200+ cows, farms with 1 to 199 cows were 3.61 times more likely to be in the high category (Table 3.5).

Table 3.4. Significance of independent variables in bivariate and multivariate logistic and linear regressions for the prediction of a farm being classified into the 'High' category for sporeformer counts from spore pasteurized bulk tank milk (80ºC (176ºF), 12 min) and association with bulk tank somatic cell counts

Independent Variable	Sporeformers*		BtSCC*	
	Bivariate	Multivariate	Bivariate	Multivariate
Milking time				
Number of audible squawks	-	-	-	-
Number of cows observed	-	-	-	-
Number of milking unit falloffs	-	-	-	-
Number of milking unit kickoffs	-	-	-	-
Use of hose to spray down milking units between cows[1]	-	-	-	-
How often milking units are sprayed down between cows[2]	-	-	-	-
How many people are milking each milking	-	-	-	-
How many people are milking each day	-	-	-	-
How many people are milking each week	-	-	-	-
How many people are milking each month	-	-	-	-
Is the California Mastitis Test used[3]	-	-	-	-
Is the California Mastitis Test used at freshening[4]	-	-	+	+ (0v2, 1v2)
Is the California Mastitis Test used after the appearance of abnormal milk[4]	-	-	+	-
Percent of cows with dirty udders in milking area[5]	+	+ (3v2, 3v1)	+	+(3v1)
Area leading to milking area cleanliness[6]	-	-	-	-
Holding area cleanliness[6]	-	-	-	-
Is holding area cleaned during each milking[4]	-	-	-	-
How often is the holding area cleaned[7]	-	-	-	-
How is the holding area cleaned[8]	-	-	-	-
Parlor Equipment				
Is the hose a garden hose or larger diameter used for cleaning[9]	-	-	+	-
Is treated water used in hoses to spray down equipment[4]	+	-	+	-
What is the treated water treated with[10]	-	-	-	-
Is the parlor deck washed down[4]	-	-	+	-
How often is the parlor deck washed down[2]	-	-	-	-
Is a hose or plumbed-in water used to wash the parlor deck[11]	-	-	+	-
Is treated water used on the parlor deck[4]	-	-	-	-
Are inflations changed on a schedules[3]	-	-	-	-
How often are inflations changed[12]	-	-	-	-
Are non-inflation rubber goods changed on a schedule[3]	-	-	+	-
How often are rubber goods changed[13]	-	-	+	-
How often is equipment serviced[13]	-	-	-	-
Is recycled water used on the farm[4]	-	-	+	-
Where is recycled water used[14]	-	-	-	-
Housing Hygiene				
Percent of cows with dirty udders in the housing area[5]	+	-	+	-
Method of scraping housing area[15]	-	-	+	-
How often are lactation pens cleaned daily[16]	-	-	-	-
Are the stalls that cows rest in cleaned as often as the housing area[4]	-	-	-	-
How many times are the stalls cleaned[17]	-	-	-	-
Is the area around the water tank cleaned as often as the housing area[4]	-	-	+	-
How many times daily is the water tank area cleaned[18]	-	-	+	-
Lactation area bedding type[19]	-	-	+	-
How often is bedding added to lactation area[20]	-	-	-	-
Dry cow area bedding[19]	-	-	+	-
How often is bedding added to dry cow area[20]	-	-	-	-
Maternity area bedding type[19]	-	-	+	-
How often is bedding added to maternity area[20]	-	-	-	-
Herd size[21]	+	+ (2 v1)	-	-

[1] (1 = no or n/a, 2 = yes).

[2] (0 = n/a, 1 = once or more per milking, 2 = as needed).

[3](1 = no, 2 = yes).

[4](0 = n/a, 1 = no, 2 = yes).

[5](1 = 0 to 10, 2 = 11 to 25, 3 = 26 to 100).

[6](0 = n/a, 1 = wet floor, 2 = manure on tip of hoof, 3 = manure touching dew claw).
[7](0 = n/a, 1 = 1x day, 2 = 2x day, 3 = 3x day or more).
[8](0 = n/a, 1 = scraper, 2 = shovel, 3 = skid steer, 4 = hand scraper, 5 = other).
[9](0 = n/a, 1 = garden, 2 = larger).
[10](0 = n/a, 1 = softener, 2 = chlorine and softener, 3 = iodine, 4 = UV light).
[11](0 = n/a, 1 = hose, 2 = plumbed).
[12](ICM = individual cow milkings, 1 = weekly, 2 = every 10 days, 3 = every 2 weeks, 4 = monthly, 5 = every 60 days, 6 = every 90 days, 7 = every 4 months, 8 = every 5-11 months, 9 = yearly, 10 = < equal to 1,000 ICM, 11 = 1,001 to 2,000 ICM, 12 = > 2,000 ICM, 13 = as needed).
[13](0 = n/a, 1 = 1x+ per month, 2 = 1x+ per year, 3 = as needed).
[14](0 = n/a, 1 = somewhere).
[15](1 = alley scrapers, 2 = skid steers, 3 = shovel, 4 = barn cleaner, 5 = hand scraper, 6 = other).
[16](1 = up to and equal to 1x per day, 2 = 2x per day, 3 = 3x+ per day).
[17](1 = 0 to 3x, 2 = 4x+ or pasture).
[18](1 = 0x and 1x, 2 = 2x, 3x+ per day or as needed, 4 = n/a).
[19](1 = sand, 2 = hay or straw, 3 = shavings or sawdust, 4 = other (manure solids, pasture, bedding combinations, or none).
[20](1 = 1x+ per day, 2 = 1x+ per week, 3 = 1x+ per month, 4 = as needed, none, or n/a).
[21](1 = 1 to 199 cows, 2 = 200+ cows).
[22*] + = significant association ($P \leq 0.10$), - = nonsignificant, ( ) indicate statistical comparison between differing levels within a specific factor.

Table 3.5. Maximum likelihood estimates of logistic regression coefficients (β), standard errors, adjusted odds ratios, and 95% confidence intervals for management factors associated with classification into the high farm category (≥ 3 log cfu/mL for spore pasteurized (80°C (176°F), 12 min) milk at any day of a 21 day refrigerated storage period at 6°C)

Factor	Factor Level	β	Standard Error	Odd Ratio (95% CI)
Intercept	-	-0.80	0.50	-
Percent dirty udders in milking area	0 to 10	-	-	-
	11 to 25	0.54	0.61	1.71 (0.51, 5.68)
	> 25	1.15	0.57	3.15 (1.02, 9.727)
Herd size	1 to 199	1.28	0.51	3.61 (1.32, 9.87)
	200+	-	-	-

To further explore the importance of the two management practices found to be significant in the final model, we also assessed the distribution of the psychrotolerant sporeformer MPN among the different ranges for 'herd size' and 'percent dirty udders in the milking area'. In contrast to sporeformer numbers in D21 SP-treated milk, MPN data represent the number of sporeformers found in the raw bulk tank milk. Herds with 1 to 199 cows had significantly lower (P = 0.0003) MPN than herds with ≥200 cows (1.32 and 1.87 mean MPN/mL, respectively; Figure 3.2). However, 70% of farms that had 1 to 199 cows were categorized with high sporeformer numbers in D21 SP-treated milk; 30% were categorized as low. For farms with herds with 200+ cows, 43% of farms were categorized as high and 57% were categorized as low (Figure 3.2). For the factor 'percent of cows with dirty udders in the milking area', farms that had > 25% of cows in the milking parlor with dirty udders had significantly higher (P = 0.0238) MPN than farms that had ≤ 25% of cows with dirty udders (2.28 mean MPN/mL for farms with > 25% of cows with dirty udders and 0.78 and 0.61 mean MPN/mL for farms with 0 to 10% and 11 to 25%, respectively). Of farms that had 0 to 10% of cows with dirty udders, 54% were categorized as high and 46% were categorized as low. For farms with 11 to 25% of cows with dirty udders, 65% of farms were categorized as high and 35% were categorized as low. For farms that had > 25% of cows with dirty udders, 77% of farms were categorized as high and 23% were categorized as low (Figure 3.2). 'Percent dirty udders' appeared to have a greater influence on MPN levels than 'herd size'. MPN levels in bulk tank milk between farms classified into the two herd sizes differed by 0.55 MPN/mL whereas the MPN level for farms with > 25% of cows with dirty udders was 1.50 MPN/mL higher than for farms with 0 to 10% of cows with dirty udders and 1.67 MPN/mL higher than for farms with 11 to 25% of cows with dirty udders.

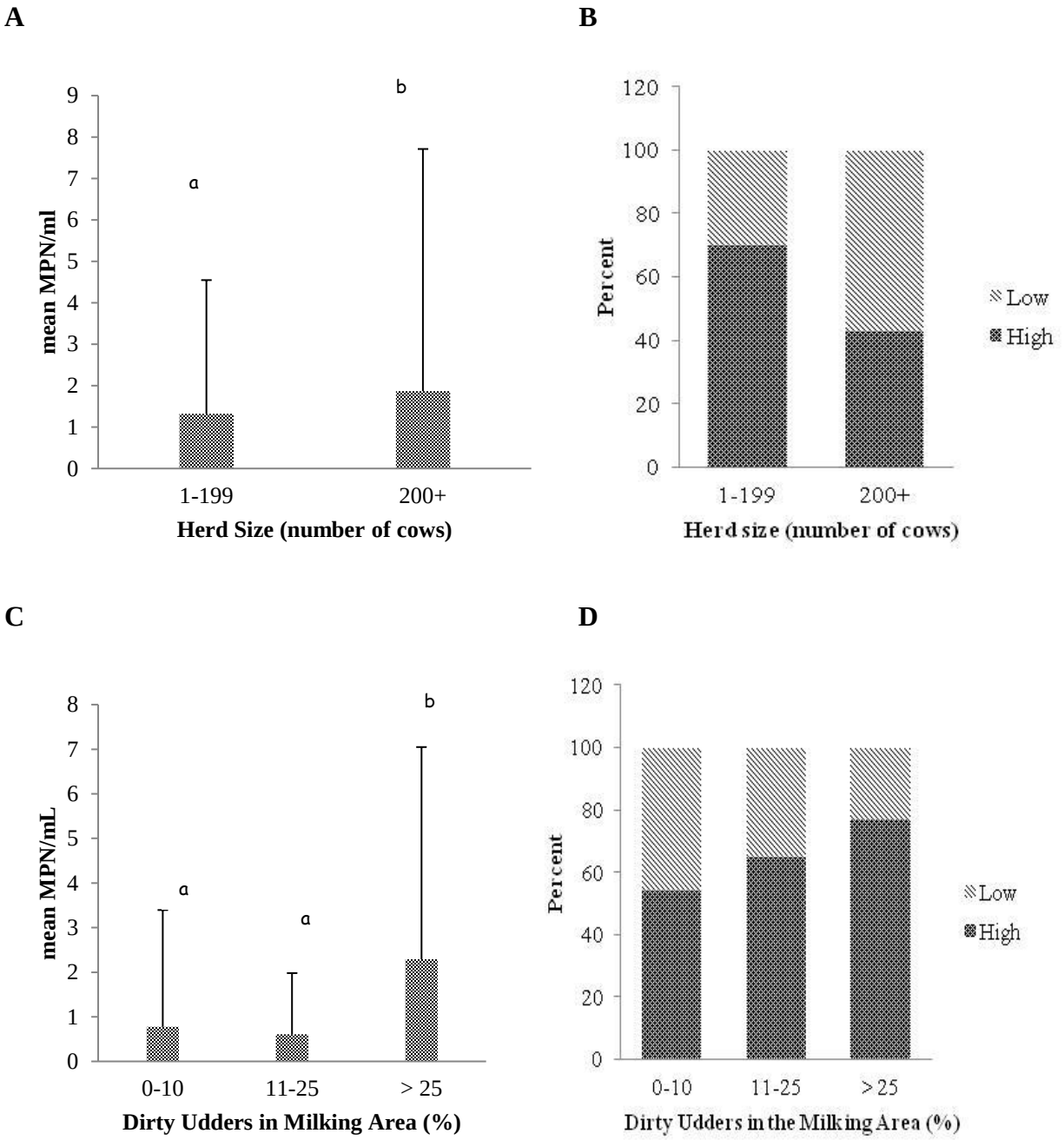

Figure 3.2. Relationships between management factors significantly associated with sporeformer counts after D21 of refrigerated storage and sporeformer levels in bulk tank milk. (2A) One way ANOVA of MPN for farms with 1 to 199 cows or 200+ cows. (2B) Distribution of high and low category farms for different herd sizes. (2C) One way ANOVA of MPN for farms with 0 to 10%, 11 to 25%, and > 25% cows with dirty udders in the milking parlor. (2D) Distribution of

high and low category farms for the different percentages of dirty udders (0 to 10%, 11 to 25%, and > 25%). Different superscript letters (a, b) between factor groups indicates a significant difference in the mean MPN/mL ($P < 0.05$).

It is important to note that our study data was limited by the questions included on our surveys (Table 3.4) and thus, we may have not captured information about other important management factors that may potentially influence psychrotolerant sporeformer levels in bulk tank milk.

Farm management practices associated with high somatic cell counts in bulk tank milk overlap with management practices associated with farms producing milk classified in the high category after SP treatment

Bivariate analyses (with logBtSCC count for each farm as the outcome) were performed for all 47 farm management factors, resulting in the following 17 factors significantly associated with BtSCC: 'is the California Mastitis Test used at freshening', ' is the California Mastitis Test used after the appearance of abnormal milk', ' percent of cows with dirty udders in the milking area', 'is a garden hose or larger diameter hose used for cleaning' (as an indicator of water flow used in the parlor), 'is treated water used in hoses to spray down equipment' , 'is the parlor deck washed down', 'is a hose or plumbed-in water used to wash the parlor deck', 'are non-inflation rubber goods changed on a schedule', 'how often are rubber goods changed', 'is recycled water used on the farm', percent of cows with dirty udders in the housing area', 'is the area around the water tank cleaned as often as the housing area', 'how many times daily is the water tank area cleaned', 'method of scraping housing area', 'lactation area bedding type', 'dry cow area bedding type', and 'maternity area bedding type' (Table 3.4). 'Percent dirty udders in housing area' was removed from further analyses due to its correlation with 'percent dirty udders in the milking area', which was used for analysis instead. After multivariate analysis, two factors remained in the model: 'percent dirty udders in the milking area' ($P < 0.10$) and 'use of the California Mastitis Test at freshening' ($P < 0.10$) (Table 3.4). Therefore, the final explanatory

model indicated that more than 25% of cows observed with dirty udders in the milking area and lack of use of the California Mastitis Test at freshening significantly influenced BtSCC levels on farms.

DISCUSSION

Our study is the first to quantify associations between dairy farm management practices and post-heat-treatment performance of refrigerated fluid milk. Multivariate analysis identified two factors ('percent of dirty udders in the milking parlor' and 'herd size') significantly associated with the likelihood of a farm having a high sporeformer level in their milk (≥ 3 log cfu/mL at any day post-SP). These results suggest that adjustments to current cow hygiene practices may improve raw milk quality as well as pasteurized shelf-life performance.

Standard raw milk quality parameters show differences between raw milk that does and does not show bacterial growth after spore pasteurization

Thirty seven percent of the raw milk samples collected in this study showed limited or no bacterial growth following SP treatment and D21 of refrigerated storage. These data suggest that a considerable proportion of commercially produced raw milk in NYS already could be processed into fluid milk products that show minimal microbial spoilage due to psychrotolerant sporeformer growth. This finding is important as growth of psychrotolerant sporeformers is a major cause of fluid milk spoilage in the U.S. (Boor, 2001; Durak et al., 2006).

Raw milk with considerable bacterial growth after SP (classified as 'high category') showed significantly higher counts for some standard raw milk quality tests (i.e., PI, PBC) when compared to milk that classified into the 'low category'. However, numerical differences in results between these tests were relatively low (< 1 log) and R^2 values for correlations between

standard raw milk quality tests and bacterial counts at D21 post-SP also were not very high (< 0.38), suggesting limited ability of current raw milk tests to predict post-SP milk performance as measured by psychrotolerant sporeformers counts. Not surprisingly, our data indicate that standard raw milk tests (BtSCC, raw milk SPC, PBC, and PI) can identify raw milk that is characterized by overall lower quality and that poor quality raw milk may perform less well than high quality milk after SP treatment. These conclusions are consistent with results from previous studies, which reported a lack of strong predictive power for raw milk microbiological tests currently used in the dairy industry (e.g., PI, PBC, sporeforming bacteria counts) for pasteurized product shelf-life (Boor et al., 1998; Martin et al., 2011). These previous studies examined raw milk microbiological quality data and corresponding microbiological performance data over shelf-life from commercial HTST pasteurized fluid milk products collected over 1 year periods from four NYS milk processors.

In addition to standard raw milk quality tests, we also evaluated bulk tank milk quality using an MPN method for psychrotolerant sporeformers. Psychrotolerant sporeformer MPN differed significantly between milk samples categorized into the 'low' and 'high' categories; MPN values between these two categories differed by more than 10 fold, which was the largest test result difference observed between high and low category milk samples. Further, the proportion of SP-treated milk samples with MPN < 0.01 was significantly larger among the samples in the low category as compared to the high category. While time consuming, requiring 21 d incubation at 6°C, the MPN test allows quantification of psychrotolerant sporeformers, which are a diverse group of organisms, including many *Paenibacillus* spp., some *Bacillus* spp. (e.g., *B. weihenstephanensis*), and *Virdibacillus* spp. With the goal of reducing detection and quantification time for critical milk spoilage organisms, a qRT-PCR assay for specific detection

of *Paenibacillus* spp. has been developed (Ranieri et al., 2012). Unfortunately, this assay does not detect psychrotolerant *Bacillus* and *Viridibacillus* spp. Our results suggest that a rapid test that detects a diverse array of psychrotolerant sporeformers in raw milk could allow for improved prediction of the performance of HTST pasteurized milk processed from this raw milk.

Bacillus and Paenibacillus spp. are the predominant sporeformers isolated after spore pasteurization

Bacillus spp. and *Paenibacillus* spp. were the predominant spoilage organisms isolated from the spore pasteurized milk in the study reported here. *Bacillus* spp. were more commonly isolated than *Paenibacillus* spp. throughout this study. This result is in agreement with previous work that showed the majority (87%) of sporeformers isolated from dairy farm environment samples (bedding, feed, manure, soil, water, and bulk tank milk) were *Bacillus* spp. (Huck et al., 2008). The proportion of isolates classified as *Paenibacillus* was significantly higher in milk at D14 and D21 post-SP as compared to milk at DI and D7. These results are consistent with previous studies of pasteurized fluid milk spoilage patterns, which also reported increasing isolation rates of *Paenibacillus* spp. over 21 d of refrigerated storage (Fromm and Boor, 2004; Ranieri et al., 2009; Ranieri and Boor, 2010). Previous work has shown that a majority of *Paenibacillus* spp. isolated from pasteurized milk have the ability to grow at low temperatures, whereas only a few *Bacillus* spp. typically grow at low temperatures (Ivy et al., 2012).

Bacillus and *Paenibacillus* spp., including many of the same species found here, have been isolated from commercially pasteurized milk and milk products in the U.S. (Fromm and Boor, 2004; Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012), Europe (Schmidt et al., 2012; Lücking et al., 2013), and Africa (Aouadhi et al.,

2013). *Bacillus* and *Paenibacillus* spp. also have been identified as the most common aerobic sporeformers in environmental samples from dairy processing facilities and dairy farms (Huck et al., 2007b; Huck et al., 2008; Ivy et al., 2012; Schmidt et al., 2012). Overall, these findings indicate that the psychrotolerant sporeformer populations found on farms represent spoilage organisms that are relevant in commercial products.

The proportion of isolates classified as *Bacillus* spp. and *Paenibacillus* spp. did not differ between high and low category farms. Whereas *Paenibacillus* spp. have been reported as more commonly having cold growth abilities than *Bacillus spp.*, our data support that cold growing *Bacillus* spp. are present in raw milk and present considerable potential for fluid milk spoilage (e.g., *B. weihenstephanensis)*. Categorization of sporeformer subtypes isolated in this study into clades associated with cold growth phenotypes (Ivy et al., 2012) showed a numerically larger proportion of isolates from the high category farms classified into clades associated with the cold growth phenotype as compared to isolates from the low category, however, some isolates from milk classified in the low category also grouped with cold growth clades (i.e., *B. weihenstephanensis*; *P. graminis*, *P. odorifer*, and *P*. cf. *peoriae*). These observations suggest that isolates within a given clade may be diverse with regard to their ability to grow at low temperatures and indicate that further work is needed to identify specific genetic determinants responsible for cold growth capabilities.

On-farm management practices influence the likelihood of high sporeforming spoilage bacteria levels in bulk tank milk

Based on the multivariate analysis of sporeformer data, we identified two management factors that were significantly associated with the likelihood of a farm producing milk that shows

considerable bacterial growth during refrigerated storage after SP treatment. Specifically, farms with a high percent of cows with dirty udders in the milking parlor and farms with less than 200 cows were more likely to have raw milk that showed considerable bacterial growth after SP. Despite the observation that larger farms were not as likely to be in the 'high' category as smaller farms, our data indicated that, in this study, larger farms had slightly higher (< 1 MPN/mL) MPN levels than smaller farms. The sporeforming bacteria present in the larger farm bulk tank samples appear to have been less capable of growing to high numbers at 6^{o}C than the microbes present in the smaller farm bulk tank samples. These findings suggest that the presence of specific microorganisms that are capable of growing to high levels under refrigeration conditions is a better predictor of D21 post-SP sporeformer numbers than the absolute number of sporeformers initially present in the bulk tank.

Other groups have examined relationships between farm size and milk quality. For example Ingham et al. (2011) reported that larger dairy farms in Wisconsin had significantly lower raw milk SPC and SCC than smaller farms. Another study reported that dairy farmers with smaller herd sizes were more likely to have management styles described as 'clean and accurate' than as 'quick and dirty' (Barkema et al., 1999). However, this study also reported that the quality of management practices used to decrease BtSCC did not differ between the two management styles, suggesting a weak relationship between herd sizes and milk quality, although this point was not explicitly tested in the study. Increasing herd size alone is unlikely to be a practical management choice for reducing sporeformer numbers in bulk tank milk. As a management factor, 'herd size' is likely a proxy for multiple farm variables, including economic resources (e.g., labor or bulk tank type and cooling ability) available for milk quality efforts.

The second management factor identified as a risk factor for farm classification into the high category was 'percent dirty udders'. This management factor not only lends itself to intervention, but was also found to have a significant effect on psychrotolerant sporeformer MPN in raw milk, with farms with a higher percent of dirty udders having substantially higher MPN. The importance of udder cleanliness for psychrotolerant spore contamination of raw milk is logical as sporeforming bacteria have been found throughout the dairy farm environment including in bedding, feed, manure, soil, and water (Scheldeman et al., 2005; Huck et al., 2008) and hence can easily be transferred to the udder and into raw milk. Previous studies suggested that adopting more hygienic practices to ensure clean udders prior to milking may be feasible, economical, and relatively simple. For example, Vissers and colleagues (2007) reported a 100 fold difference (3 to 300 mg/L) in the quantity of dirt present on udders between farms with good hygiene practices and farms with poor hygiene practices. Another study found that poor teat-end cleanliness was associated with higher bacteria counts in bulk tank milk (Elmoslemany et al., 2009). Further, adopting the hygienic practice of cleaning teats with moist paper towels reduced the number of *Clostridia tyrobutyricum* spores isolated from raw milk (Magnusson et al., 2006).

To determine whether the same or different practices affect both a traditional milk quality parameter (i.e., BtSCC) as well as parameters related to the presence of psychrotolerant sporeformers, we also performed a multivariate analysis for associations of management factors with BtSCC. This analysis showed that a lower percent of 'dirty udders in the milking parlor' and 'use of a California Mastitis Test at freshening' both were significantly associated with lower BtSCC. Barkema et al. (1999) also reported that farms with low BtSCC had better observed animal hygiene scores. Associations between use of CMT specifically at freshening and lower BtSCC can be explained by the fact that freshening is a crucial time to mitigate

intramammary infections and subsequent SCC levels (Oliver et al., 2003). Another study reported that SCC was lower for cows that were assigned to a CMT-based treatment program for subclinical mastitis as compared to cows that were assigned to the control program (no CMT usage) (Lago et al., 2012).

Overall, our data indicate that milking time practices used to control mastitis on dairy farms (specifically those practices affecting udder cleanliness) may also help control psychrotolerant sporeformer levels in bulk tank milk. Therefore, focusing on cow cleanliness may yield the dual benefits of achieving raw milk quality economic incentives for producers and improved pasteurized product performance for consumers.

CONCLUSIONS

As the dairy industry ships milk farther and longer between farm of origin and location of consumption (Womble et al., 2008), controlling the presence of sporeforming spoilage organisms throughout the milk production and processing continuum (Huck et al., 2007a) is essential for producing high quality, long lasting fluid milk products. This study identified dairy farm management practices related to milking time hygiene that may simultaneously lower BtSCC on dairy farms as well as psychrotolerant sporeformer levels in bulk tank milk. Our results suggest that on-farm adjustments in management decisions specifically focused on udder cleanliness may directly impact the shelf-life of pasteurized fluid milk. Our data reported here represent the microbiological quality of bulk tank milk obtained at a single time point on each farm. Additional studies are needed to determine if farms can consistently produce raw milk that does not show bacterial growth following heat treatment.

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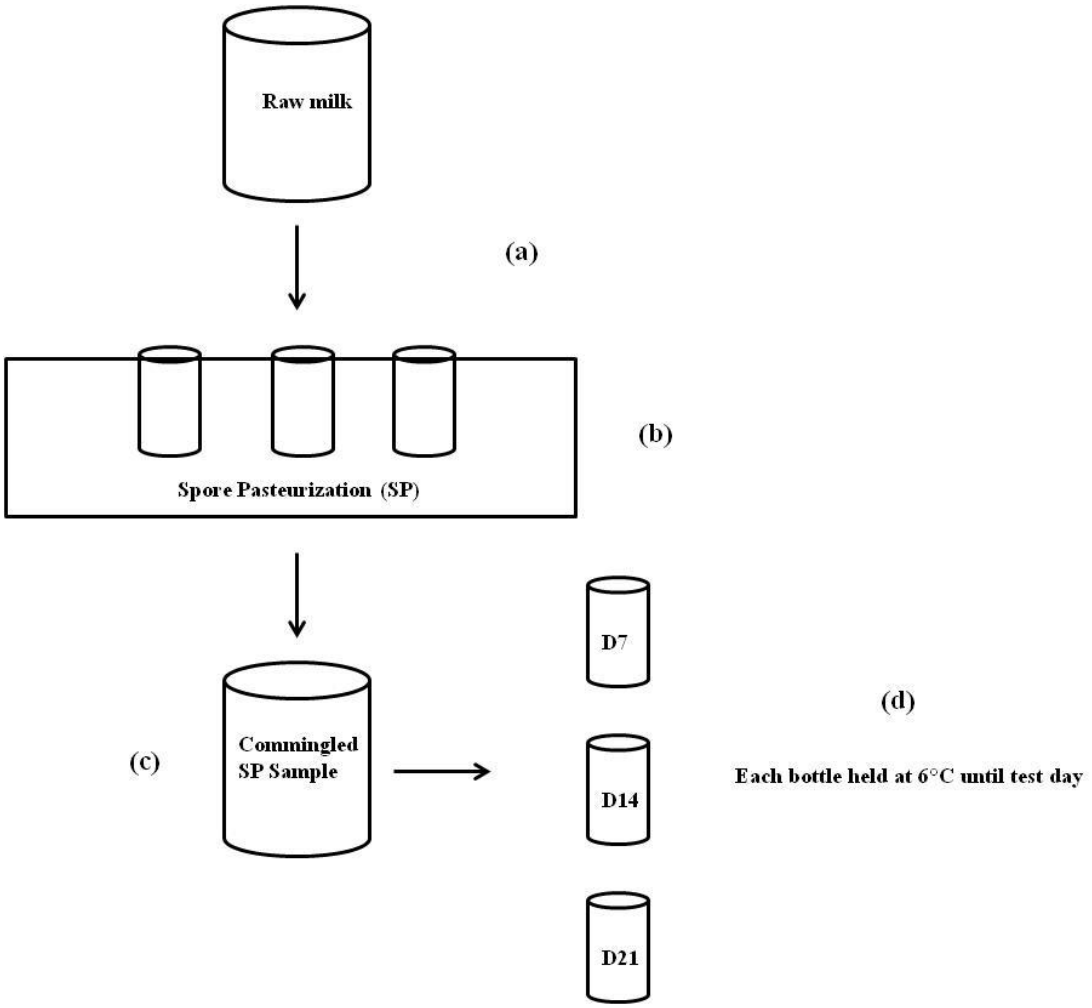

Supplemental Figure 3.1. Preparation of milk samples for post-spore pasteurization (SP) microbiological testing. (a) After removal of aliquots for raw milk microbiological analyses, the remaining raw milk was distributed equally into three sterile 250 mL glass bottles. (b) SP was performed by heat treating the three bottles at 80°C (176°F) for 12 min followed by immediate cooling on ice to 6°C. (c) Post cooling, milk from all three bottles was commingled into a sterile 500 mL glass bottle and aliquots were removed for post-SP microbiological testing for day initial (DI), including MPN analyses. (d) Remaining post-SP milk was divided equally into three sterile bottles and held at 6°C for microbiological testing at days 7 (D7), 14 (D14), and 21 (D21) post-SP.

CHAPTER 4

LONGITUDINAL ASSESSMENT OF DAIRY FARM MANAGEMENT PRACTICES ASSOCIATED WITH THE PRESENCE OF PSYCHROTOLERANT BACILLALES SPORES IN BULK TANK MILK ON 10 NEW YORK STATE DAIRY FARMS

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ABSTRACT

The ability of certain sporeforming bacteria in the order Bacillales (e.g., *Bacillus* spp., *Paenibacillus* spp.) to survive pasteurization in spore form and grow at refrigeration temperatures results in product spoilage and limits HTST pasteurized fluid milk shelf-life. To facilitate development of strategies to minimize contamination of raw milk with psychrotolerant Bacillales spores, we conducted a longitudinal study of 10 New York State dairy farms, which included yearlong monthly assessments of bulk tank raw milk psychrotolerant spore contamination frequency and levels along with administration of questionnaires to identify farm management practices associated with psychrotolerant spore presence over time. Milk samples were first spore pasteurized [80°C (176°F) for 12 min] and then analyzed for sporeformer counts on the initial day of spore pasteurization (SP), and after refrigerated storage (6°C) for 7, 14, and 21 d after SP. Overall, 41% of samples showed sporeformer counts of > 20,000 cfu/mL at day 21 post-SP with *Bacillus* and *Paenibacillus* spp. as predominant causes of high sporeformer counts. Statistical analyses identified three management factors (more frequent cleaning of the bulk tank area, the use of a skid steer to scrape the housing area, and segregating problem cows during milking) that were all associated with lower probabilities of d 21 Bacillales spore detection in SP-treated bulk tank raw milk. Our data emphasize that appropriate on-farm

measures to improve overall cleanliness and cow hygiene will reduce the probability of psychrotolerant Bacillales spore contamination of bulk tank raw milk, allowing for consistent production of raw milk with reduced psychrotolerant spore counts, which will facilitate production of HTST-pasteurized milk with extended refrigerated shelf-life.

Key words: *Bacillus* spp., *Paenibacillus* spp., spoilage, management practice

INTRODUCTION

Bacterial spoilage is the predominant limiting factor in the shelf-life of pasteurized fluid milk (Boor, 2001; Durak et al., 2006). In the absence of post-pasteurization contamination of the product, sporeforming bacteria are the predominant residual organisms in pasteurized fluid milk. Gram-positive *Bacillus* spp. and *Paenibacillus* spp. form heat-resistant spores able to withstand high temperature short time (**HTST**) [72°C (161°F), 15 s] pasteurization commonly used for fluid milk processing (Collins, 1981; Fromm and Boor, 2004; Ranieri et al., 2009) with certain strains able to grow at refrigerated storage temperatures, resulting in milk spoilage (Washam et al., 1977; Huck et al., 2008). Previous work identified *Bacillus* spp. as predominant sporeformers early in the refrigerated shelf-life of fluid milk, while the prevalence of *Paenibacillus* increased over shelf-life (Fromm and Boor, 2004; Ranieri et al., 2009). Characterization of bacterial isolates showed that only a few specific *Bacillus* species (e.g., *B. weihenstephanensis*) but many *Paenibacillus* species are able to grow during refrigerated storage of milk. These sporeforming spoilage bacteria, via metabolic activities, can lead to loss of product quality (e.g., curdling, off odors or flavors) (Ageitos et al., 2007; Dutt et al., 2009). Controlling these psychrotolerant Bacillales spores in raw milk is critical to the extension of fluid milk shelf-life.

Elimination of psychrotolerant sporeforming bacteria is challenging as they are found throughout the general environment (e.g., soil, decaying matter, plant surfaces, mammalian digestive tracts) (Gilliam et al., 1984; Gilliam, 1985; Sarkar, 1991; Fredrickson and Onstott, 1996; Nicholson, 2002) and more specifically, the dairy farm environment. For example, *Bacillus* spp. were frequently isolated on Scottish dairy farms, from both the dairy farm environment and raw bulk tank milk (Crielly et al., 1994). *Paenibacillus* spp. have also been

isolated from silage, dairy cow feed concentrate, and raw milk (Vaerewijick et al., 2001; te Giffel et al., 2002; Scheldeman et al., 2004). Also, in a recent study in the US, sporeforming psychrotolerant bacteria have been isolated from samples taken along the dairy processing continuum, from milk trucks to packaged final products; identification of the same bacterial subtypes in both raw and pasteurized milk samples suggests that pasteurized fluid milk spoilage can result from spores that originate from raw milk on the farm (Huck et al., 2007b).

A previous cross-sectional study on the relationship between on-farm management practices and psychrotolerant Bacillales spore levels in bulk tank milk from 99 dairy farms in New York State (**NYS**) found that practices related to milking time hygiene may influence the levels of spores present in bulk tank raw milk (Masiello et al., 2014). Specifically, improved udder cleanliness was linked to reduced psychrotolerant Bacillales spore counts (Masiello et al., 2014). Due to the cross-sectional nature of this study, it did not evaluate how spore levels or management practices may change over time on a dairy farm. Exploring the factor of time is important as previous work has identified associations between the isolation of psychrotolerant *Bacillus spp*. from fluid milk and specific seasons (Phillips and Griffiths, 1986; Griffiths and Phillips, 1990; Sutherland and Murdoch, 1994). For example, studies on both bulk tank and pasteurized milk in Scotland found that psychrotolerant sporeforming bacteria were more commonly isolated in the summer and autumn months (Phillips and Griffiths, 1986; Griffiths and Phillips, 1990). Additionally, another Scottish study exploring seasonal occurrence found that psychrotolerant *Bacillus spp*. were most prevalent during the summer and autumn months while mesophilic (growth at 30°C for 72 h) *Bacillus spp*. often dominated in the winter months (Sutherland and Murdoch, 1994).

We hypothesized that specific farm-associated factors, including on-farm management practices and seasonal factors, may be associated with psychrotolerant spore contamination of bulk tank raw milk. With the goal of future development of specific recommendations for management of spore numbers in bulk tank milk, the objectives of this study were: (i) to assess and characterize psychrotolerant spore contamination patterns in bulk tank milk across and within dairy farms over time; and (ii) evaluate the associations of on-farm management practices and seasonal factors with psychrotolerant spore contamination in bulk tank milk over the course of one year.

MATERIALS AND METHODS

Farm Selection

A longitudinal study with monthly sampling repetitions was conducted on 10 NYS dairy farms from February 2011 to February 2012 (excluding December 2011). Herds were selected from the Quality Milk Production Services (QMPS) (College of Veterinary Medicine, Cornell University, Ithaca, NY) program clientele at four different QMPS locations, representing four different regions in NYS, including regions surrounding Ithaca (3 farms), Canton (2 farms), Cobleskill (3 farms), and Geneseo (2 farms). Farms were selected based on willingness to participate and previous classification of psychrotolerant Bacillales spore levels, as described by Masiello et al. (2014). Specifically, five farms previously identified as having a 'high' psychrotolerant Bacillales spore level (≥ 3 log cfu/mL after 21 days of 6°C storage post-spore pasteurization [80°C, 12 min]) and five farms previously identified as having a 'low' psychrotolerant Bacillales spore level (< 3 log cfu/mL over all 21 days of 6°C storage post-spore pasteurization) were included in the study. All participants were fully informed of the design of

the study, the nature of the data being collected and their future use, and were aware that the study was voluntary. Each participant signed an informed consent document acknowledging the above items.

Farm characteristics

Herd sizes ranged from 32 to 1,368 cows, with a mean herd size of 535 ± 480 cows. Cows were housed in freestalls (65%), tiestalls (25%), or on pasture (10%). Cow breeds included Holstein (60%) and multiple or mixed breeds (40%). The number of lactating cows per farm ranged from 26 to 909 (mean of 371 ± 326 lactating cows), with farms milking between 2 and 4 times daily (30% at 2x, 60% at 3x, and 10% at 4x). Average milk production across farms was 10,821 kg (23,857 lbs) ± 2,714 kg (5,984 lbs) per cow per year and ranged from 4,468 kg (9,850 lbs) to 14,250 kg (31,415 lbs).

Survey Design

The survey used in this study was modified from an existing QMPS survey that included questions on herd health, housing cleanliness, equipment maintenance, milking time procedures, and medication usage (Table 4.1). The modified survey included 4 pages previously used by QMPS and 1 page that focused on specific potential risk factors deemed relevant for spore contamination (Table 4.1); the supplemental 1-page survey had previously been used in a cross-sectional study (Masiello et al., 2014). The survey was designed to be administered verbally to the participant.

Table 4.1. Summary of farm management practice data collected from surveys administered each month for a year (n = 12 sampling visits per farm) for 10 farms in New York State.

Factor[1]	**Description**[2]
Herd information	Visiting QMPS technician, DHIA testing, number of milking cows, number of milkings per day, breed of cows, average milk production per year, is the herd a closed herd, does the farm purchase replacement cows, number of milking cows observed
Seasonal factors	Month of sampling
Herd health	Percent cows with unhealthy teats, percent cows with injured teats, Selenium added to feed, Selenium injection given, number of clinical cases of mastitis per month, number of lactating cows treated for mastitis each month, lactating cow mastitis treatment used, number of cows that died in the prior year due to mastitis, *Escherichia coli* vaccination given to milking cows, treatment given to dry cows, timing of dry off for cows, problem cows sold, California Mastitis Test used
Cow hygiene[3]	Cow hygiene score in milking area, percent dirty udders in the milking parlor, cow hygiene score in housing area, percent dirty udders in the housing area, tail docking, udder clipping
Milking routine[3]	Gloves worn during milking, average milking time, use of udder prep, type of udder prep, products used in udder prep, are udders washed, product used to wash udders, how are udders dried, use of forestripping, problem cows segregated during milking, problem cows milked last, problem cows milked with separate unit, number of milking claw falloffs, number of milking claw kickoffs, number of milking claw falloffs into manure, number of milkers each day, number of milkers each week, number of milkers each month
Housing[3]	Cleanliness of lactation cow housing, type of lactation cow housing, type of bedding used in lactation cow housing area, cleanliness of dry cow housing, type of dry cow housing, type of bedding used in dry cow housing area, cleanliness of maternity pen, type of maternity pen housing, type of bedding used in maternity pen, type of cleaning method for housing areas, frequency of cleaning of housing areas
Sanitation[3]	Milking unit sanitation, type of sanitizers used, milking hose alignment, milking units sanitized between milking groups, frequency of milking unit sanitation, parlor deck washed, frequency of parlor deck wash
Equipment	Make of milking claw, manufacturer of milking claw, vacuum shut off of milking units, rubber equipment changed on a schedule, frequency of equipment service

[1] General categories of farm management practices and temporal factors used in data analyses.
[2] Summary of data collected from survey questions answered at each monthly sampling.
[3] Factors included on one-page survey.

Survey Administration and Bulk Tank Sampling

Each location had a designated and trained QMPS technician who administered the survey and collected samples. Technicians administering the survey were trained to obtain objective answers without being leading, to focus on quantitative/numeric answers, and to follow training guides for any necessary subjective scores or observations (e.g., percent dirty udders in the milking parlor). Farm visits occurred every month from February 2011 to February 2012, except for December 2011; both bulk tank sample collection and survey administration were performed at each farm visit.

Bulk tank raw milk at each farm was sampled using two sterile dip vials and one National Dairy Herd Information Association (DHIA; Verona, WI) vial; vials were immediately stored on ice packs in a cooler and held at ≤ 6ºC. Bulk tank raw milk samples (250 mL in each of two vials) were shipped overnight to the Milk Quality Improvement Program (MQIP) laboratory (Department of Food Science, Cornell University, Ithaca, NY) in Styrofoam coolers packed with ice packs. Sample temperature was recorded immediately upon arrival at the laboratory. Any samples with temperatures > 6°C upon arrival were rejected and the farm was re-sampled. DHIA vials were shipped directly from QMPS locations to Dairy One (Ithaca, NY) for bulk tank somatic cell count (BtSCC) analysis using a Fossomatic FC ESCC automated SCC reader (Foss Inc., Hillerød, Denmark).

NYS Monthly Weather Data Collection

Average monthly weather data for each of the four QMPS regions were obtained from the North East Regional Climate Center (Cornell University, Ithaca, NY). The data were collected via weather stations located in Ithaca, Canton, Cobleskill, and Avon (less than 10 miles [16 km]

from Geneseo, NY). The monthly data collected included maximum temperature (°F), minimum temperature (°F), average temperature (°F), total precipitation (in), total snowfall (in), and average snow depth (in). Monthly weather data were obtained for the period of February 2011 to February 2012, excluding December 2011.

Microbiological Evaluation of Milk Samples

For each farm sampling, the two sample vials (250 mL each) were commingled into one sterile 500 mL glass bottle. Raw milk samples were completely inverted 25 times prior to removal of an aliquot for microbiological analyses, which included (i) total bacteria count (TBC) on Standard Plate Count Agar (SPCA) (Difco, BD Diagnostics, Franklin Lakes, NJ) as described by Laird et al. (2004); (ii) Psychrotrophic Bacteria Count (PBC) (Laird et al., 2004) and (iii) Preliminary Incubation (PI) count (Martin et al., 2011b).

The remaining raw milk was distributed equally among three sterile 250 mL glass bottles for spore pasteurization (SP), performed by heat treating each of the three bottles (~150 mL each) at 80°C (176°F) for 12 min, followed by immediate cooling on ice. After cooling to 6°C, the samples in each bottle were commingled into a sterile 500 mL glass bottle. The bottle was fully inverted 25 times and two 100 µl aliquots were plated on SPCA to determine the "Day Initial" (DI) mesophilic spore count (after incubation at 32°C for 48 h). The remaining SP milk samples were then split equally into the three 250 mL glass bottles previously used during spore pasteurization and held at 6°C for microbiological testing (TBC) at 7, 14 and 21 d post-SP. Additionally, a modified five tube most probable number (MPN) method (Davidson et al., 2004) was used to quantify psychrotolerant Bacillales spore levels below or around the detection limit of direct plating (10 CFU/ml). The MPN method was performed on SP samples as follows: 10

mL of SP milk was aliquoted into each of five sterile screw capped tubes, 1 mL of SP milk was aliquoted into each of five sterile screw capped tubes containing 9 mL of sterile skim milk broth (SMB) (1:10 dilution) and finally, 0.1 mL of SP milk was aliquoted into each of five sterile screw capped tubes containing 9.9 mL of sterile SMB (1:100 dilution). Each of the fifteen tubes was vortexed and then incubated at 6°C for 21 d prior to spiral plating on SPCA. Plates were evaluated for presence or absence of growth after 48 h of incubation at 32°C. MPN data were interpreted using a five tube MPN table (Davidson et al., 2004).

Bacterial Isolate Collection

Bacterial colonies representing visually distinct morphologies (typically 1 to 4 colonies per plate) were selected and streaked for purity on Brain Heart Infusion (BHI) agar (Difco) from SPCA plates used for bacterial enumeration on each sampling date (DI, d 7, d 14, d 21). BHI agar plates were subsequently incubated at 32°C for 24 h. Pure cultures were grown overnight in BHI broth at 32ºC prior to freezing in 15% glycerol at -80ºC. A total of 746 isolates were collected using this approach. Isolate information can be found at www.foodmicrobetracker.com.

Molecular Characterization and Identification of Isolates

Sequencing of a 632 bp fragment of *rpoB,* as described by Huck et al. (2007a), was used for characterization and species identification of Bacillales spore isolates obtained from SP milk samples plated on DI, d 7, 14, and 21. Briefly, cultures were streaked for colony isolation from frozen stock onto BHI agar and grown at 32ºC for 24 h. A sterile toothpick was used to sample an isolated colony, which was used for lysate preparation and subsequent PCR to amplify a 740 bp *rpoB* fragment, following previously described procedures (Ivy et al., 2012). After verifying

amplification by gel electrophoresis, DNA fragments were treated with Exonuclease I (Affymetrix, Santa Clara, CA) and Shrimp Alkaline Phosphatase (New England Biolabs, Ipswich, MA), as described by Dugan et al., 2002, and bidirectional sequencing with PCR primers was performed by the Life Sciences Core Laboratory Center (Cornell University, Ithaca, NY) using Sanger sequencing. *rpoB* sequence alignment and assignment of *rpoB* allelic types (AT) was performed as described by Ivy et al. (2012); *rpoB*-based classification to genus and species was performed as described by Miller et al. (2015b). For isolates that could not be classified to genus and species by *rpoB* sequencing, partial 16S rDNA sequencing was used to facilitate genus and species classification as previously described (Ivy et al., 2012). If definitive species identification was not possible but isolates closely resembled a named species, species were classified as "cf." (defined as "confer"; e.g., *P.* cf. *peoriae*) where cf. denotes an uncertain identification.

Data Analyses

Bacillales spore count data and raw milk test data were logarithmically transformed before analyses. Pearson's product-moment correlations were calculated between all milk tests (raw milk TBC, PBC, PI, BtSCC, DI through d 21 Bacillales spore count, and MPN) within R studio (version 0.98.484) (Studio, 2012) using R statistical software (R x64 v 3.0.2) (R Team, 2014). Correlation coefficients were tested for significance using t-tests, and Holm-Bonferroni multiple testing correction was performed. Additionally, a Fisher's Exact Test was performed in SAS (version 9.3) (Cary, NC) to explore the difference in number of *Bacillus* and *Paenibacillus* spp. isolates obtained each month.

To assess potential associations between d 21 Bacillales spore data and management practices and weather, a logistic regression was used. This analysis was performed within R

studio (version 0.98.484) using R statistical software (R x64 v 3.0.2) (Miller et al., 2015a). Using the 'lme4' (Bates et al., 2013) and 'dplyr' (Wickham and Francois, 2016) packages, the best fitting model was determined via forwards-stepwise selection-based on the Akaike Information Criteria (AIC) (Akaike, 1998). The AIC for the trivial random-effects only model was calculated and compared to the AICs of models with one explanatory variable added (i.e., a candidate model). If none of the candidate models had a lower AIC than the starting model, the selection terminated. Otherwise, the candidate model with the lowest AIC was chosen as the current model, and the process of adding one explanatory variable began again. 'Farm' was included as a random factor. Interactions were not considered, and autocorrelation was not assessed.

RESULTS

Milk from 10 New York State Farms Showed a Wide Range of Bacterial Counts Following SP and Subsequent Incubation at 6°C

A total of 120 bulk tank raw milk samples were collected across the 10 participating farms, using monthly sampling for a year. After SP treatment and subsequent incubation at 6°C, each participating farm had at least one sample with a bacterial count > 1.0 $\log_{10}$cfu/mL after 21 days of refrigerated storage (Table 4.2). Overall mean bacterial counts (inclusive of all farms for all months; n = 120 milk samples) increased over the 21 days post-SP; with mean bacterial counts of 2.00 ± 1.0 $\log_{10}$ cfu/mL (DI), 2.1 ± 1.1 $\log_{10}$ cfu/mL (d 7), 3.4 ± 1.8 $\log_{10}$ cfu/mL (d 14), and 4.4 ± 2.2 $\log_{10}$ cfu/mL (d 21).

Table 4.2. Average Bacillales sporeformer count (log_{10}cfu/mL) after 21 days of refrigerated storage at 6°C in spore-pasteurized (80°C [176°F] for 12 min) bulk tank milk samples collected from 10 NYS dairy farms over 1 year.

Farm	Month[1,2]													
	Feb	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**Oct**	**Nov**	**Jan***	**Feb***	**Number of samples with detectable sporeformers at day 21**	**Average d 21 count (log_{10}cfu/mL) among samples with detectable sporefomers**
A	ND	**5.8**	**5.2**	1.3	2.3	1.0	**4.4**	ND	**6.0**	**6.4**	ND	ND	6/10	4.0
B	3.1	**4.7**	2.8	2.9	1.7	**5.2**	ND	**5.2**	**7.6**	2.7	**4.9**	**7.0**	9/10	4.3
C	**6.0**	**6.8**	**7.5**	**5.8**	**5.0**	**4.7**	**6.7**	**7.1**	**7.6**	**5.1**	**7.2**	**7.3**	10/10	6.4
D	**5.6**	**7.7**	**7.4**	**5.7**	**6.0**	**6.9**	ND	**5.4**	**7.6**	ND	**6.1**	2.6	8/10	6.1
E	ND	ND	ND	1.0	ND	1.6	1.0	1.3	2.2	**5.2**	3.7	3.8	6/10	2.5
F	**5.6**	ND	ND	1.0	ND	ND	**4.9**	ND	ND	**5.3**	ND	**5.2**	5/10	4.4
G	**6.4**	**7.2**	**6.5**	**5.8**	**6.2**	1.3	2.0	**4.9**	**6.8**	**7.3**	**5.4**	**6.8**	10/10	5.5
H	3.9	ND	2.2	1.5	ND	ND	ND	ND	2.4	1.0	1.5	ND	4/10	2.1
I	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.7	ND	1/10	2.7
J	1.0	1.5	3.9	**5.3**	3.5	ND	ND	3.0	**5.8**	1.3	1.0	2.2	8/10	2.8
Number of samples with detectable sporeformers at day 21	7/10	6/10	7/10	9/10	6/10	6/10	5/10	6/10	8/10	8/10	8/10	7/10		
Average d 21 count (log_{10}cfu/mL) among samples with detectable sporefomers	4.5	5.6	5.1	3.4	4.1	3.5	3.8	4.5	5.8	4.3	4.1	5.0		

[1] Numbers in bold indicate bacterial counts above 20,000 cfu/mL (4.30 logs), the Pasteurized Milk Ordinance (PMO) limit for Grade 'A' fluid milk.

[2] ND indicates no sporeformers were detected in the sample.

The d 21 post-SP bacterial counts varied by both month and farm (Table 4.2). Of the 120 samples analyzed, 37 showed no signs of psychrotolerant sporeformer growth over 21 days, while 49 milk samples (41%) had d 21 post-SP bacterial counts > 20,000 cfu/mL (> 4.3 log) (Table 4.2). Of the samples where psychrotolerant spores were present, the monthly average d 21 counts (across all farms combined) ranged from 3.4 $\log_{10}$cfu/mL to 5.8 $\log_{10}$cfu/mL during the year-long study period, while the farm average d 21 counts (across all months combined) ranged from 2.1 $\log_{10}$cfu/mL to 6.4 $\log_{10}$cfu/mL. For two study farms (H and I), all 12 milk samples for each farm had d 21 post-SP bacterial counts < 20,000 cfu/mL (< 4.3 log). On the other hand, one farm (C) had d 21 post-SP bacterial counts > 4.5 $\log_{10}$cfu/ml for all 12 samples collected.

The geometric mean BtSCC among the 120 raw milk samples was 208,300 cells/mL (5.3 ± 0.3 $\log_{10}$cells/mL). Raw milk TBC ranged from 419 to 13,900,000 cfu/mL, with a geometric mean raw milk TBC of 10,000 cfu/ml (4.0 ± 1.0 $\log_{10}$cfu/mL). The PI counts for all milk samples ranged from 756 to 22,000,000 cfu/mL, with a geometric mean PI count of 52,000 cfu/mL (4.7 ± 1.2 $\log_{10}$cfu/mL). PBC for raw milk ranged from below detection limit (< 10 cfu/mL) to 13,300,000 CFU/mL, with a geometric mean PBC of 900 cfu/mL (3.0 ± 1.4 $\log_{10}$cfu/mL) among the 108 samples with detectable counts. The MPN for psychrotolerant Bacillales spores (determined on SP-treated milk incubated at 6°C) ranged from < 0.01 (18 samples) to > 24 MPN/mL (6 samples), with a mean MPN of 0.9 ± 2.2 MPN/mL among the 99 samples with detectable MPN.

The correlation analysis showed that MPN was significantly ($P < 0.05$) correlated with DI through d 21 Bacillales spore counts, as well as raw milk BtSCC, TBC, and PI counts, with correlation of determination ranging from $r^2 = 0.14$ to $r^2 = 0.35$ (Supplemental Table 4.1).

Furthermore, in addition to MPN, d 21 sporeformer counts were significantly correlated with DI through d 14 Bacillales spore counts and raw milk BtSCC, with coefficient of determination ranging from $r^2 = 0.13$ to $r^2 = 0.30$ (Supplemental Table 4.1). With correlation coefficients all ≤ 0.35, we observed no strong correlation between MPN or d 21 Bacillales spore count with any of the raw milk tests.

Bacillus and Paenibacillus Were the Predominant Bacillales Genera Isolated from SP-Treated Milk Samples

Initial analysis of *rpoB* AT data for 746 isolates allowed us to identify a total of 442 representative bacterial isolates obtained from the 120 milk samples (12 milk samples per farm) at different time points after SP treatment (Table 4.3). Representative isolates were obtained by including only a single isolate if multiple isolates with the same AT were obtained from the same sample and the same day of refrigerated storage. The majority of these representative isolates were classified as *Bacillus* spp. (70.8%; 313/442 isolates) or *Paenibacillus* spp. (28.3%; 125/442 isolates; Table 4.3). *Lysinibacillus* spp. and *Viridibacillus arvi/arenosi* accounted for 0.9% (4/442) of the total isolates (Table 4.3).

Table 4.3. Numbers and prevalence of Bacillales genera and species obtained, at the initial day (DI) and d 7, 14, and 21 of refrigerated storage at 6°C, from spore-pasteurized bulk tank milk samples collected from 10 New York State farms over one year

Bacterial genus and species[1]	**DI**	**d 7**	**d 14**	**d 21**	**Total no. isolates**	**% of isolates**
Bacillus spp. (total)	100	93	67	53	313	70.8
B. aerophilus	0	0	1	0	1	0.2
B. cf. *aerophilus*	8	3	0	0	11	2.5
B. cereus sensu lato	3	1	6	3	13	2.9
B. clausii	1	0	0	1	2	0.5
B. horneckiae	1	0	0	0	1	0.2
B. licheniformis	34	43	12	10	99	22.4
B. pumilus	36	29	13	13	91	20.6
B. safensis	6	5	4	1	16	3.6
Bacillus sp.	1	0	1	0	2	0.5
B. subtilis sensu lato	9	3	3	2	17	3.8
B. weihenstephanensis	1	9	27	23	60	13.6
Paenibacillus spp. (total)	6	25	49	45	125	28.3
P. amylolyticus sensu lato	1	0	1	2	4	0.9
P. cf. *cookie*	2	0	2	0	4	0.9
P. cf. *peoriae*	3	10	23	23	59	13.4
P. graminis	0	1	2	2	5	1.1
P. odorifer	0	14	20	15	49	11.1
Paenibacillus sp.	0	0	1	3	4	0.9
Lysinibacillus sp.	0	0	0	1	1	0.2
Viridibacillus arvi/arenosi	0	0	2	1	3	0.7
Total	**106**	**118**	**118**	**100**	**442**	**100**

[1] Sensu lato = in the broad sense; cf. = unspecified identification, resembling the named species.

The predominant *Bacillus* and *Paenibacillus* species among the characterized isolates were *B. licheniformis, B. pumilus,* and *B. weihenstephanensis* and *P*. cf. *peoriae* and *P. odorifer* (Table 4.3). For isolates obtained from milk at DI and d 7 after SP, 86.2% and 13.8% were classified as *Bacillus* (193/224) and *Paenibacillus* (31/224), respectively; for isolates obtained from milk at d 14 and 21, 56.1% (120/214) and 43.9% (94/214) were classified as *Bacillus* or *Paenibacillus*, respectively. The number of *Bacillus* and *Paenibacillus* spp. isolated significantly differed by month ($P < 0.001$; Fisher's Exact Test). Months with the highest proportion of *Bacillus* isolates obtained were November, September, and August with 94, 86, and 79%, respectively (Supplemental Table 4.2). The highest proportion of *Paenibacillus* isolates were obtained in March, April, and February with 55, 40, and 36%, respectively (Supplemental Table 4.2).

The *rpoB* sequence data allowed for further classification of isolates to specific subtypes (*rpoB* AT); a total of 89 unique AT were found among the characterized isolates representing all farms. The most prevalent AT were: AT-1 (*B. licheniformis*; 61 isolates), AT-3 (*B. weihenstephanensis*; 37 isolates), AT-20 (*B. pumilus*; 32 isolates), AT-6 (*B. licheniformis*; 31 isolates), AT-179 (*P*. cf. *peoriae*; 25 isolates), and AT-337 (*B. pumilus*; 22 isolates). For 9 of the study farms, isolates representing the same genus were obtained from samples collected during different months (Figure 4.1). For the same 9 farms, isolates with the same AT were obtained from samples collected during at least two different months. For example, the majority of AT-337 isolates (15 of the 22 isolates with this AT) were obtained from farm C; on this farm, AT-337 isolates were obtained in 11 of the 12 months (Figure 4.1). On the other hand, AT-1 was re-isolated from 9 farms, consistent with previous reports that this is the most common *Bacillus* AT in raw milk (Ranieri et al., 2009). The single farm (Farm I; See Figure 4.1) that did not have any

ATs re-isolated during different months was also the farm with the least number of representative isolates obtained (n = 6 isolates).

Farm No..	Re-isolated Genus	Re-isolated Species	Re-isolated rpoB-AT (no. of isolates) for isolates representing the re-isolated genus or species by month											
			Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Jan*	Feb*
A	*Bacillus*	*licheniformis*	1 (1)	1 (1)	1 (1)			1 (1)		1 (2)				
		pumilus		137 (1)								137 (1)		
B	*Bacillus*	*licheniformis*	1 (3), 6 (1)	6 (2)			1 (1), 6 (1)	6 (1)			6 (1)	1 (2), 6 (2)	1 (2), 6 (2)	1 (4), 6 (1)
		pumilus	20 (3), 62 (1), 68 (1), 144 (1)	20 (2), 62 (1)	62 (1), 68 (2)		20 (2)	144 (1)	20 (1), 144 (1), 337 (1)		20 (1)	20 (4), 144 (1)	20 (1), 144 (1), 337 (2)	337 (1)
		subtilis s.l.											66 (1)	66 (1)
		weihenstephanensis			75 (1)		3 (2)			75 (1)	3 (1), 75 (1)		3 (2)	75 (1)
	Paenibacillus	cf. *peoriae*							199 (1)		199 (1)			
		odorifer											27 (1)	27 (1)
C	*Bacillus*	*licheniformis*	1 (2), 6 (1)			1 (2)	1 (1)	1 (1)	1 (2)	6 (2)	6 (1)	1 (2), 6 (1)	1 (1)	1 (1), 6 (2)
		pumilus	20 (1), 337 (1), 373 (1)	337 (1)	20 (1), 337 (1)	337 (2)	20 (2), 337 (1)	20 (1), 337 (3)		337 (1), 373 (1)	337 (1)	337 (2)	337 (1)	337 (1)
		weihenstephanensis								3 (1)	75 (2)		3 (1), 75 (2)	
	Paenibacillus	cf. *peoriae*	157 (2), 179 (1), 199 (1)	157 (1), 179 (1), 199 (1)	179 (2)	179 (1)	179 (2), 334 (1)		179 (1)		179 (2)		199 (1)	334 (1)
		odorifer	15 (2), 27 (1)	7 (1), 15 (2)	27 (1)							27 (1)	7 (1), 15 (1), 27 (1)	7 (1)
D	*Bacillus*	*licheniformis*					1 (1)	1 (1)			1 (1)			
		weihenstephanensis	3 (3)	3 (1)	3 (1)	3 (2)	3 (3)			3 (2)	3 (1)	3 (1)		3 (1)
	Paenibacillus	cf. *peoriae*	199 (1)		199 (3)		199 (1)							
		odorifer		15 (1)									15 (1)	15 (1)
E	*Bacillus*	*licheniformis*				1 (1)			1 (1)		1 (1)			
		weihenstephanensis	3 (1)							3 (1)				
F	*Bacillus*	*cereus* s.l.											410 (1)	410 (1)
		licheniformis						1 (1)	1 (1)					
	Paenibacillus	cf. *peoriae*								179 (1)	179 (1)			
G	*Bacillus*	cf. *aerophilus*			135 (1)		135 (1)		135 (1)					
		licheniformis	1 (3)	1 (1)	1 (1)	1 (1)	1 (1), 6 (2)	1 (1)	1 (1), 6 (1)	6 (1)	1 (1)	1 (1), 6 (2)	1 (1)	1 (1)
		pumilus	20 (2), 68 (1)	68 (1)		68 (1)		20 (2)	62 (1)	20 (1)		62 (1)	20 (1)	20 (1), 62 (1)
		safensis					378 (1)					106 (1)	106 (1)	378 (1)

		subtilis s.l.					66 (1), 211 (1)		66 (2), 211 (1), 331 (1)		331 (1)			
		weihenstephanensis	3 (1)							3 (1)	3 (3)	3 (1)		
	Paenibacillus	cf. *peoriae*		179 (1)	179 (3)	179 (1)	170 (2)	170 (1)	170 (1)	179 (1)	170 (1)			179 (3)
		odorifer	21 (1), 74 (1)	74 (1)		21 (1), 74 (1)							21 (1)	
		sp.	77 (1)				77 (1)			77 (1)				
H	*Bacillus*	*licheniformis*				1 (1)				1 (1)				
	Paenibacillus	cf. *peoriae*	179 (1)			179 (1)					179 (1)			
I	-	-												
J	*Bacillus*	cf. *aerophilus*		135 (1)										135 (1)
		cereus s.l.	410 (1)				410 (1)	410 (1)						
		licheniformis	1 (1)		1 (1)	6 (1)	1 (1)	1 (1)			1 (1), 6 (2)		1 (1), 6 (1)	
		pumilus	20 (1)	20 (1)	144 (1)							20 (1), 144 (1)		
		safensis			254 (1)							254 (1)		
		weihenstephanensis				3 (1)			3 (1)	3 (1)	3 (1)			

Figure 4.1. Genus, species, and allelic types (AT) of Bacillales isolates that were isolated over multiple months from spore-pasteurized (80°C [176°F] for 12 min) bulk tank milk samples collected from 10 New York State dairy farms.

'Frequency of tank area cleaning', 'skid steer scrapers used', and 'problem cows segregated' are significantly associated with probability of d 21 Bacillales spore detection in SP-treated bulk tank milk

After variable selection for the logistic regression, three management factors were significantly associated (all at $P < 0.01$) with the probability of d 21 Bacillales spore detection in SP-treated bulk tank milk including: (i) frequency of bulk tank area cleaning (ii) whether skid steer scrapers were used to clean the housing area, and (iii) whether problem cows were segregated during milking (Table 4.4). Two more variables, 'stall cleanliness' and 'other stall cleaning method used' were retained in the final model, but were not significant. There was no significant association between either weather factors or month of sampling and the probability of d 21 Bacillales spore detection in SP-treated bulk tank milk. For the factor 'frequency of bulk tank area cleaning', each additional cleaning was associated with a 14.6-fold decrease in the odds of d 21 Bacillales spore detection. Use of skid steer scrapers was associated with a 150.2-fold decrease in the odds of d 21 Bacillales spore detection, while segregation of problem cows during milking time was associated with a 44.3-fold decrease in the odds of d 21 Bacillales spore detection.

Table 4.4. Farm management practices significantly ($P < 0.01$) associated with probability of Bacillales spore detection in spore-pasteurized bulk tank milk samples after 21 days of refrigerated storage (6°C)

Variable	Variable level	Effect estimate (log odds) [1]	Effect estimate (fold change) [2]
Frequency of tank area cleaning	n/a – continuous variable	-2.682	14.6^{-1}
Skid steer scrapers used	No (reference)	0	1
	Yes	-5.012	150.2^{-1}
Problem cows segregated	No (reference)	0	1
	Yes	-3.790	44.3^{-1}

[1] The log odds estimate indicates, for a one unit change in a continuous variable or change from the reference for a categorical variable, the consequent predicted additive change in $\log \frac{p}{1-p}$, where p is the probability of a sample showing d 21 presence of psychrotolerant Bacillales spores at ≥ 10 spores/mL.

[2] The fold change estimate indicates, for a one unit change in a continuous variable or change from the reference for a categorical variable, the consequent predicted multiplicative change in the odds $\frac{p}{1-p}$, where p is the probability of a sample showing d 21 presence of psychrotolerant Bacillales spores at ≥ 10 spores/mL.

DISCUSSION

This longitudinal study quantified associations between dairy farm management practices and psychrotolerant Bacillales spore levels in heat-treated refrigerated milk over the course of one year. Multivariate logistic regression analysis and correlation analysis identified three factors (frequency of bulk tank area cleaning, using a skid steer to clean the housing area, and segregating problem cows during milking) that were significantly associated with the probability of psychrotolerant Bacillales spore detection at 21 d of refrigerated storage after SP. These significant factors identify general cleanliness components of dairy farm management and suggest that adjustments to current cleaning practices as well as management of milking time factors may allow for better management of psychrotolerant Bacillales spoilage organisms and, therefore, pasteurized milk shelf-life performance.

Some Farms Consistently Produce Raw Milk with Low Levels of Psychrotolerant Bacillales Spore Contamination, Despite the Fact that Current Milk Quality Tests Poorly Correlate with Psychrotolerant Bacillales Spore Contamination

Psychrotolerant Bacillales spores have been identified as key factors limiting the shelf-life of HTST fluid milk that is not exposed to post processing contamination (Boor, 2001; Durak et al., 2006). If present, psychrotolerant Bacillales spores typically cause spoilage of HTST milk by day 17 to 21 of refrigerated storage at 6°C (Fromm and Boor, 2004). A previous cross-sectional study showed that bulk tank raw milk from 37.4% of 99 farms contained such low levels of psychrotolerant Bacillales spores that no spore outgrowth was observed in 100mL of heat treated milk over 21 days of storage at 6°C post SP treatment (using the same methodology applied in the current study) (Masiello et al., 2014). However, this previous study did not determine whether farms consistently produce raw milk with low psychrotolerant Bacillales

spore levels, which is a concern as variation in bacterial counts has been reported for bulk tank raw milk. Hayes and colleagues showed that TBC in a given farm can vary considerably; for example, bulk tank milk from 8 of 13 farms experienced occasional "spikes" with TBC ranging from 4.2 to 5.8 $\log_{10}$cfu/mL (Hayes et al., 2001). Consistent with previous data on TBC, our study here supports that many farms show monthly variation in bulk tank raw milk contamination with psychrotolerant Bacillales spores. For example, four bulk tank raw milk samples from farm F showed high bacteria counts at day 21 post SP (all > 4.5 logs), while the other 8 samples showed counts < 1.0 log at day 21 post SP (≤ 10 cfu/ml). Among the 10 farms enrolled, two (farms H and I) were consistently capable of producing milk that showed limited (< 3 logs) or no bacterial growth following SP treatment and 21 d of refrigerated storage. These results suggest that psychrotolerant Bacillales spore levels can be managed to be consistently low. This indicates that it should be possible to develop a raw milk supply that could dependably be processed into fluid milk products that would show minimal microbial spoilage due to psychrotolerant Bacillales spore outgrowth.

Appropriate diagnostic tests are key tools that allow producers to manage raw milk quality. Traditionally, spore count methods are not routinely used to monitor raw milk quality; in particular, a psychrotolerant Bacillales spore count test would be difficult to use routinely, as the time to result would be at least 10 days (these tests involve a 10 day incubation of plates at 6°C). While we found significant correlations between commonly used raw milk quality tests and bacterial counts at d 21 post-SP, the coefficient of determination values were not very high (ranging from 0.00 to 0.13), implying a limited ability of the raw milk tests to predict the d 21 post-SP bacterial counts (Supplemental Table 4.1). These results are consistent with a previous cross-sectional study, which also reported low coefficient of determination values between the

raw milk quality tests and bacterial counts at d 21 post-SP from bulk tank samples from 99 NYS dairy farms (Masiello et al., 2014). Additionally, a previous study in New York examining, over one year, associations between raw milk microbiological quality parameters and microbiological performance data over shelf-life for HTST pasteurized fluid milk products found that there was little predictive power for raw milk microbiological tests currently used in the dairy industry (e.g., PBC, or PI count) and pasteurized product shelf life (Martin et al., 2011). On the other hand, the study reported here found modest correlations between psychrotolerant Bacillales spore MPN counts and bacterial counts at d 21 post-SP. Although the MPN test seems to be the most useful test for quantification of psychrotolerant Bacillales spores, it is time and labor intensive. While work has been done to develop a real-time quantitative PCR assay for specific detection of *Paenibacillus* spp. (Ranieri et al., 2012), this assay does not detect psychrotolerant *Bacillus* spp. or other psychrotolerant Bacillales that represent different genera (e.g., *Viridibacillus* spp.). Our results suggest that a rapid molecular test capable of quantitative detection of all psychrotolerant Bacillales would facilitate management of raw milk quality for improved HTST milk shelf-life; increased availability of genomic information for psychrotolerant Bacillales spores (Moreno Switt et al., 2014) may provide an opportunity for the development of these types of tests.

Bacillus and Paenibacillus Are Consistently the Predominant Bacillales Genera Isolated from Milk Samples after SP

Bacillus spp. and *Paenibacillus* spp. were the predominant Bacillales spores isolated from SP milk in the current study. The overall frequency of isolation of *Bacillus* and *Paenibacillus* spp. significantly differed between months during the yearlong study. Our data

indicated that the highest proportion of *Bacillus* spp. were isolated in the late autumn and winter, while the highest proportion of *Paenibacillus* spp. was isolated in the late winter and spring. Previous work from a 1994 Scottish study assessing seasonal occurrences of *Bacillus* spp. in raw milk found that the incidence of *B. pumilus*, *B. licheniformis*, and *B. subtilis*, classified in the study as mesophilic sporeformers, was highest during the winter (specifically from November to March) (Sutherland and Murdoch, 1994). These observations suggest that certain sporeformer isolates may be more likely to be isolated during cooler months and indicated that further work is needed to identify the relationship between climate factors and specific psychrotolerant Bacillales genera associated with bulk tank raw milk.

In the current study, *Bacillus* represented the genus most commonly isolated from samples representing 11 of the 12 sampling months. By comparison, in a previous cross-sectional study evaluating associations between farm practices and psychrotolerant Bacillales spores in bulk tank milk, 71.4% and 26.4% of all isolates (obtained from SP milk over 21 d of storage at 6 °C) were classified as *Bacillus* spp. and *Paenibacillus* spp., respectively (Masiello et al., 2014). These findings are consistent with previous studies, which have shown that *Bacillus* and *Paenibacillus* are the predominant Bacillales genera isolated from commercially HTST pasteurized milk and milk products all over the globe, including the US (Fromm and Boor, 2004; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012; Masiello et al., 2014; Miller et al., 2015a), Europe (Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994; Schmidt et al., 2012; Lücking et al., 2013) and Africa (Aouadhi et al., 2013). Additionally, *Bacillus*, and to a lesser extent *Paenibacillus* spp., have been regularly isolated from environmental samples taken from dairy farms (Huck et al., 2007b; Ivy et al., 2012; Schmidt et al., 2012). In one previous work, the majority (87%) of spores isolated from the dairy farm environment (bedding, feed,

manure, soil, water, and bulk tank milk) were classified as *Bacillus* spp., while 13% of isolates were classified as *Paenibacillus* spp. (Huck et al., 2008). The percentage of isolates classified as *Paenibacillus* spp. in the current study were higher in milk at d 14 and 21 after SP compared with milk at DI and d 7. These results are consistent with previous studies of spoilage patterns in pasteurized milk that reported increasing isolation rates of *Paenibacillus* spp. over 21 d of refrigerated storage (Fromm and Boor, 2004; Ranieri et al., 2009; Ranieri and Boor, 2009; Masiello et al., 2014) as well as previous work which has shown that, among dairy-associated isolates, the majority of *Paenibacillus* spp. but very few *Bacillus* spp. have the ability to grow at low temperatures (Ivy et al., 2012). Overall, these data indicate that the predominant Bacillales spores in raw milk are typically *Bacillus* spp., which include psychrotolerant as well as mesophilic and thermophilic strains (Huck et al., 2008; Masiello et al., 2014; Miller et al., 2015b), while cold storage after both experimental and commercial HTST pasteurization selects for *Paenibacillus* spp., which are predominantly psychrotolerant, as well as psychrotolerant *Bacillus* strains.

On-Farm Management Practices Related to Cleanliness Influence the Probability of Psychrotolerant Bacillales Spore Detection in Bulk Tank Milk

We identified three management factors significantly associated with d 21 sporeformer presence in raw bulk tank milk. More frequent cleaning of the bulk tank area, the use of a skid steer to scrape the housing area, and segregating problem cows during milking were all associated with lower probabilities of d 21 Bacillales spore detection in SP-treated bulk tank raw milk. While these factors are derived from specific survey questions, it is likely that they are all proxies for general farm cleanliness rather than explicit management factors which can guarantee low Bacillales spore presence. For example, the use of a skid steer for housing area cleaning

may (i) directly affect the cleanliness of cows entering the milking parlor and therefore affect spore presence and/or (ii) act as a proxy for the farm staff's priority given to cleanliness and therefore also affect spore presence.

The association of general cleanliness management factors with d 21 sporeformer presence is consistent with previous studies that have identified that on-farm cleanliness factors, relating to a cow's daily life (from housing to milking), can impact bacterial counts in bulk tank milk. Soil and manure have been previously identified as risk factors for milk contamination with psychrotolerant Bacillales spores in the general dairy farm environment (Vissers et al., 2007; Huck et al., 2008). In a Dutch study among 24 dairy farms, researchers observed a significant correlation between spore concentrations in bulk tank milk and in manure samples collected from both pastures and housing areas, noting that a higher spore concentration in manure correlated with a higher *B. cereus* concentration in bulk tank milk (Vissers et al., 2007). In addition to farm environment cleanliness, cow hygiene (via teat-end cleanliness) has been shown to be associated with bacteria counts in bulk tank milk (Elmoslemany et al., 2010). In a study of 153 herds on Prince Edward Island (Canada), both the total aerobic and preliminary incubation counts in bulk tank milk samples were significantly associated with the amount of dirt on teats, indicating that dirtier teats were associated with higher counts (Elmoslemany et al., 2010). Previous studies also support the importance of maintaining good hygiene from farm environment to cow, indicating the effect of housing hygiene on teat cleanliness (Plesch and Knierim, 2012) and, therefore, bacteriological quality of bulk tank raw milk (Elmoslemany et al., 2010). Our data suggest that managing overall farm cleanliness (via manure/dirt contamination reduction from housing to parlor to milkhouse) is a key intervention and feasible strategy that will reduce psychrotolerant Bacillales spore presence in bulk tank raw milk.

CONCLUSIONS

Consistent control of the presence of psychrotolerant Bacillales spoilage organisms throughout milk production and the milk processing continuum is needed in order to produce high-quality and long-lasting fluid milk products. Our study identified that dairy farm management practices related to overall dairy farm cleanliness were associated with the presence of psychrotolerant Bacillales spores in bulk tank milk after 21 days at 6°C post-heat treatment. These results suggest that on-farm adjustments in management decisions focused on both environmental and cow cleanliness may have a direct impact on psychrotolerant Bacillales spore presence and hence the shelf-life of pasteurized fluid milk. As our data here included only a few environmental factors, such as temperature and rainfall, and as our data was observational; (i) additional studies on the potential associations between psychrotolerant sporeformer counts and a larger range of environmental factors (including interactions with management factors) as well as (ii) conducting experimental rather than observational work relating to on-farm management practices may be valuable to further refine strategies to allow for consistent annual production of raw milk with low psychrotolerant Bacillales spore levels.

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Supplemental Table 4.1. Coefficient of determination (r^2) between d Initial through d 21 Bacillales sporeformer counts, raw milk TBC, raw milk PI, raw milk PBC, BtSCC, and raw milk psychrotolerant Bacillales spore MPN values[1]

	BtSCC							
TBC	$r^2 = 0.17$*	**TBC**						
PBC	$r^2 = 0.06$	$r^2 = 0.44$*	**PBC**					
PI	$r^2 = 0.24$*	$r^2 = 0.51$*	$r^2 = 0.53$*	**PI**				
d Initial sporeformer	$r^2 = 0.19$*	$r^2 = 0.17$*	$r^2 = 0.06$	$r^2 = 0.10$	**D Initial sporeformer**			
d 7 sporeformer	$r^2 = 0.22$*	$r^2 = 0.08$	$r^2 = 0.08$	$r^2 = 0.15*$	$r^2 = 0.03$	**d 7 sporeformer**		
d 14 sporeformer	$r^2 = 0.13$*	$r^2 = 0.12$*	$r^2 = 0.04$	$r^2 = 0.04$	$r^2 = 0.06$	$r^2 = 0.09$	**d 14 sporeformer**	
d 21 sporeformer	$r^2 = 0.13$*	$r^2 = 0.10$	$r^2 = 0.00$	$r^2 = 0.03$	$r^2 = 0.21$*	$r^2 = 0.20$*	$r^2 = 0.30$*	**d 21 sporeformer**
sporeformer MPN	$r^2 = 0.33$*	$r^2 = 0.20$*	$r^2 = 0.08$	$r^2 = 0.30$*	$r^2 = 0.15$*	$r^2 = 0.35$*	$r^2 = 0.14$*	$r^2 = 0.20$*

[1] TBC = Total Bacteria Count; PI = Preliminary Incubation Count; PBC = Psychrotrophic Bacteria Count; BtSCC = Bulk Tank Somatic Cell Count; MPN = Most Probable Number Count.

* Indicates P<0.05

Supplemental Table 4.2. Proportion of representative[1] Bacillales isolates obtained from spore-pasteurized bulk tank milk samples collected from 10 New York State farms over one year by sampling month

Month	% *Bacillus* spp. (n)	% *Paenibacillus* spp. (n)	% Other genera[3] (n)
February	70.5 (41)	27.5 (16)	2.0 (1)
March	40.0 (16)	55.0 (22)	5.0 (2)
April	60.0 (16)	40.0 (11)	0.0 (0)
May	70.0 (18)	30.0 (8)	0.0 (0)
June	74.0 (28)	26.0 (10)	0.0 (0)
July	78.5 (22)	21.5 (6)	0.0 (0)
August	79.0 (26)	21.0 (7)	0.0 (0)
September	86.0 (25)	14.0 (4)	0.0 (0)
October	74.0 (32)	26.0 (11)	0.0 (0)
November	94.0 (33)	6.0 (2)	0.0 (0)
January[2]	68.3 (28)	29.3 (12)	2.4 (1)
February[2]	64.0 (28)	36.0 (16)	0.0 (0)
Total	**70.8 (313)**	**28.3 (125)**	**4**

[1] Representative isolates were obtained by including only a single isolate if multiple isolates with the same AT were obtained from the same sample and the same day of refrigerated storage at 6°C.
[2] Months in 2012.
[3] Other genera include *Lysinibacillus* sp. and *Viridibacillus arvi/arenosi*

CHAPTER FIVE

CONCLUSIONS

Dairy farmers and processors strive to create high quality, long lasting pasteurized fluid milk. However, these efforts can be limited by the presence of psychrotolerant spoilage organisms. By 2050, the dairy and other food industries will need to feed an increasing number of consumers in different areas of the world. Ensuring a safe and quality product with an extended shelf-life will be vital in providing nutrient-rich pasteurized fluid milk to areas of low food security. This research aimed to provide feasible strategies for the reduction of psychrotolerant spoilage organisms in pasteurized fluid milk and, therefore, the potential for extension of refrigerated shelf-life.

To accomplish this, the main goal of this work to was better understand psychrotolerant spoilage organisms associated with pasteurized fluid milk. We hypothesized that levels of psychrotolerant sporeforming organisms in fluid milk may be influenced by management decisions made on the farm. We also hypothesized that assessing coliforms capable of post-pasteurization contamination of fluid milk by more specific phylogenetic characteristics could result in enough variation that processors may need to change their methods of intervention for this group. Thus, each study focused on obtaining high quality milk through data-informed decisions during fluid milk production and processing.

In the first study, we identified that psychrotolerant coliforms introduced as PPC in fluid milk display taxonomic diversity and have varying abilities to exhibit lipolytic and proteolytic activity. Since our data suggested that certain coliform 16S strain types were repeatedly isolated in products from specific facilities, future work is needed to more explicitly identify and define sources of PPC. For example, future efforts may include exploring potential differences in cold

growth and enzymatic activity capabilities within multiple coliform genera and subtypes, thereby providing information useful for prioritizing interventions. This work demonstrated that hygiene issues within a fluid milk processing plant could lead to PPC with a diverse group of coliform contaminants that are capable of not only having a direct impact on pasteurized milk quality, but impacting the consumer's experience through cold growth and spoilage capabilities.

In the second study, we identified that dairy farm management practices related to milking time hygiene can influence psychrotolerant sporeformer levels observed in bulk tank milk. The results of this study indicated that on-farm adjustments in management, specifically focused on udder cleanliness, could directly impact the shelf-life of pasteurized fluid milk. However, the data reported from the second study used only a single time point to represent the psychrotolerant sporeformer levels on the farms. Prior literature has described the impact of season/weather on bacteria levels on dairy farms, thus suggesting that additional work would be needed to determine if any of the factors identified were confounded by seasonal trends or differences.

In the third study, we took a longitudinal approach to exploring if on-farm management decisions could directly impact psychrotolerant sporeformer levels in heat-treated milk. We identified that dairy farm management practices related to overall dairy farm cleanliness were associated with the presence of psychrotolerant Bacillales spores in bulk tank milk after 21 days at 6°C post-heat treatment. The results of the third study validated those observed during the cross-sectional second study. The third study results also suggested that on-farm adjustments in management decisions focused on both environmental and cow cleanliness may have a direct impact on psychrotolerant Bacillales spore presence; directly impacting the shelf-life of pasteurized fluid milk. Future studies are needed to assess a larger array of potential

environmental and management factors, as well as to perform experimental studies where different mitigation strategies could be tested to assess consistent annual production of raw milk with low psychrotolerant Bacillales spore levels.

These studies broaden the understanding of psychrotolerant spoilage organisms associated with pasteurized fluid milk and provide the basis for intervention strategies to feasibly achieve high quality products. We have identified several controllable factors that influence the likelihood of psychrotolerant sporeformers in pasteurized fluid milk and have explored the phylogenetic and spoilage diversity of coliforms associated with fluid milk post-pasteurization contamination. Due to the presence of these spoilage organisms throughout the general environment, the creation of a single method or technology to prevent contamination is unlikely. However, these studies show that consistently high quality pasteurized fluid milk can be achieved by making data-informed improvements to current spoilage organism reduction methods. Evaluating the cleaning protocols for dairy cow environments and the milking parlor could greatly impact the levels of psychrotolerant sporeformers observed in pasteurized fluid milk. Additionally, a more specific assessment of coliform organisms isolated from fluid milk could provide processors with information to create more effective cleaning and sanitation regimens.

In short, these studies highlight three straight-forward steps to reduce psychrotolerant spoilage organisms in fluid milk: (i) make informed decisions based on organism spoilage and growth capabilities, (ii) evaluate current practices and protocols to assess if they are assisting in achieving the end goal of high milk quality, and (iii) prioritize the investment of time and effort in farm and facility cleanliness to improve milk quality. Adopting these steps will promote

longer refrigerated shelf- life of pasteurized fluid milk, thereby allowing a nutrient rich product to reach a larger number of consumers.